Natural transformation and recombination in Helicobacter pylori

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Natural transformation and recombination in *Helicobacter pylori*

ACADEMISCH PROEFSCHRIFT

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”From a prokaryotic perspective, sexual eukaryotes like ourselves are incestuous nymphomaniacs: we do “it” far too often and almost exclusively with partners that, from a phylogenetic perspective, are essentially identical to ourselves.”

Levin, B.R. and Bergstrom, C.T. (2000) Proc Natl Acad Sci U S A 97(13): 6981-6985

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Chapter 1: general introduction

Helicobacter, transformation, and *Helicobacter* transformation

Partially adapted from:

Natural transformation in *Helicobacter pylori*: DNA transport in an unexpected way.

Smeets LC, Kusters JG (2002) Trends Microbiol 10(4):159-62.

Helicobacter pylori

The discovery of *Helicobacter pylori*

Gastric curved or spiral bacteria have first been observed in animals over 100 years ago [28, 142] and later also in humans [52, 62, 65]. These observations received little attention at the time. Attempts to isolate these unknown organisms did not succeed and examinations of gastric suction material failed to confirm the histological findings [126]. The observed bacteria were often considered to be contaminating organisms from the mouth, bowel, or gastric contents. With the improvement of endoscopic techniques, however, it became possible to obtain biopsies of the antrum during lifetime and the curved bacteria were observed in these biopsies as well. In 1975 Steer and Colin-Jones compared the epithelial differences between the normal stomach (six subjects) and 47 patients with gastric ulcers. In 80% of the patients with a gastric ulcer, bacteria were observed. These were, however, incorrectly identified as *Pseudomonas aeruginosa* [151]. Finally, in 1983 Warren and Marshall described small, curved bacilli visible on routine histology in gastric biopsies, most consistently in the antrum, in about half of routine gastric biopsy specimens. These bacteria were nevertheless "almost unknown to clinicians and pathologists alike". Thanks to a combination of clever observation (they noticed that the bacteria resembled *Campylobacter*) and careless negligence (they forgot to throw away their culture plates before an Easter holiday) they achieved for the first time to culture the organisms using *Campylobacter* culture techniques with a prolonged incubation, and isolated the bacterium that is now known as *Helicobacter pylori* [175].

The era of *Helicobacter pylori*

After the first cultivation of *Helicobacter pylori*, in 1984 the same authors published a study of 100 consecutive patients presented for gastroscopy [109]. They demonstrated small, curved bacilli in 58 gastric biopsies and cultured these bacteria from 11 patients. They noticed that the bacteria were present in almost all patients with active chronic gastritis, duodenal ulcer, or gastric ulcer. The bacterium, first considered to be a *Campylobacter* species and called *Campylobacter pylori*, now gained more attention. Its existence as well as its association with gastric inflammation were soon confirmed by others [88, 110]. It also became evident that the taxonomic classification in the *Campylobacter* genus was not correct. Sequence determination of the DNA encoding ribosomal RNA (rDNA) warranted the

classification of *Campylobacter pylori* as the first species of a new genus. In 1989, it was renamed *Helicobacter pylori* [1, 68, 136].

Pathogenesis

The scarce data on acute *H. pylori* infections suggest that the disease typically starts as an acute gastritis with nausea and epigastric pain after an incubation period of approximately six days. Within two weeks, the symptoms resolve but the infection can change into a chronic, asymptomatic infection of the antrum with gastritis [108, 115].

Soon after the discovery of *H. pylori* a strong correlation between *H. pylori* colonization and peptic ulceration was established [29, 30]. Only ten years later, in 1994, *H. pylori* research had wiped away a second dogma of modern medicine: the general belief that the main causes for peptic ulceration were stress and smoking. That year, a consensus conference convened by the National Institutes of Health of the USA concluded that *H. pylori* was a major cause of peptic ulcer disease. It recommended that peptic ulcer patients be treated to eradicate *H. pylori* from the stomach [119]. Eradication of *H. pylori* is nowadays a mainstay method of peptic ulcer treatment. However, the role of *H. pylori* in non-ulcer dyspepsia is not yet settled and is still matter of an ongoing debate [87, 107, 113, 119].

The most common presentation of *H. pylori* is a chronic infection characterized by asymptomatic gastritis, which can progress to atrophic gastritis and potentially intestinal metaplasia and gastric adenocarcinoma. It was already known that the development of intestinal metaplasia and gastric adenocarcinoma was linked to the presence of chronic gastritis and in 1991 an association between *H. pylori* and gastric adenocarcinoma was demonstrated [61, 121, 129, 159]. In 1994 the World Health Organization announced that *H. pylori* should be considered as a carcinogen for humans [81]. *H. pylori* has also been implicated in the development of other gastric neoplasms, most notably gastric **m**ucosa-**a**ssociated **l**ymphoid **t**issue lymph**oma** (MALToma) [179]. Treatment of *H. pylori* infection has been reported to lead to regression of this tumor, which makes MALToma the only human neoplasm that can be treated with antibiotics [22, 58, 59, 178]. On the other hand, there is some evidence of an inverse relationship between *H. pylori* colonization and gastroesophageal reflux (GERD), Barrett's esophagus, and adenocarcinomas of the gastric cardia and lower esophagus, suggesting that *H. pylori* might offer protection from these diseases [4, 31, 127].

Finally, statistical relationships between colonization with *H. pylori* and a large number of entries from the medical dictionary have been published, suggesting that *H. pylori*

is to blame for an extraordinary wide variety of human ailments, ranging from Parkinson's disease to late menarche and from diabetes mellitus to primary Raynoud's phenomenon. The best evidence usually is not indicative of a role for *H. pylori* in these diseases [49]. The most notorious of these allegations against *H. pylori* is probably the connection between *H. pylori* and atherosclerosis, but even in this case evidence for a causal relationship is lacking [116].

Microbiology

H. pylori is a gram-negative rod of 2.5 to 10µm long and 0.5 to 1 µm wide. The most distinctive biochemical characteristics are catalase positivity, oxidase positivity and strong urease positivity. The G+C content is 39 mol% [164]. In a Gram stain of a culture it appears as a Gram-negative, comma-shaped bacterium or as a short spiral with a maximum of three turns. In a broth culture, however, it assumes a spiral morphology with 5 to 20 turns [56]. In the stomach, *H. pylori* is present either as curved rods or as short spirals [109, 162, 175] (Fig. 1). After prolonged incubation in culture, coccoid forms appear. These coccoid bacteria are unculturable in vitro [35]. It has been suggested that that at least a subset of the coccoid forms is capable of renewed growth under appropriate conditions, and that the coccoid form is in fact a dormant growth phase that could play a role in transmission of the infection [32, 120]. However, coccoid cultures have been shown to undergo degenerative changes such as fragmentation of the DNA and RNA, protein degradation, loss of membrane potential, low concentrations of mRNA and a gradual decline in ATP content [54, 94]. The spherical form therefore most likely represents a passive morphological change after cell death.

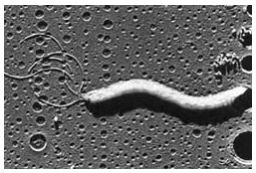
FIG. 1: *H. pylori*

H. pylori possesses four to six unipolar flagella of approximately 2,5 µm long and 30 nm thick [67]. These flagella are composed of FlaA and FlaB protein subunits [157] and posses a membranous flagellar sheath, which forms a bulb at the end [64].

Approximately half of the *H. pylori* strains carries a plasmid [67]. The size of the plasmids varies from 1.5 kbp [91] to 40 kbp [67]. No function has as yet been assigned these plasmids, and strains with or without plasmid show no difference in antibiotic resistance pattern [67]. There is no evidence for the presence of conjugational plasmids.

Laboratory culture

H. pylori is a rather fastidious organism. Culture requires microaerobic conditions (5% O_2, 5 – 7% CO_2) [69] at 30 – 40°C [85], but growth can also occur under anaerobic

conditions. After inoculation of single colony forming units (CFU) on solid media, small colorless colonies appear after 3 to 5 days. Well-adapted laboratory strains can survive for several hours at aerobic conditions, but this is not the rule for clinical isolates. Incubation of more than 5 days under normal culture conditions will result in a rapid decrease of viable bacteria; heavily inoculated solid media can become completely non-viable within 48 hours. Culture is normally performed on rich media such as Columbia base agar supplemented with 5 – 10% sheep or horse blood [165]. Also media with human blood are employed. For broth culture as well as solid media, brucella medium or brain heart infusion medium with 2 – 10% fetal calf serum or with 5 – 10% horse serum are used [165]. *H. pylori*-selective antibiotics like Skirrow's supplement (vancomycin, trimethoprim and polymyxin B) [69] or Dent's supplement (vancomycin, trimethoprim, cefsulodin and amphotericin) [51] are commonly added to the growth medium. The addition of 0,4% 2,3,5 triphenyltetrazolium chloride in solid media gives precipitation of "golden" crystals in and around the small and otherwise colorless *H. pylori* colonies, which is helpful in detection as well as in discrimination between *H. pylori* and contaminants.

Survival in acidic conditions

The normal habitat of *H. pylori*, the stomach, is designed to eliminate microbial contaminants from food, and thus provides *H. pylori* with a lack of microbial competition that is unequalled on human mucosal surfaces. The drawback is that *H. pylori* has to deal with the inhospitable environment, in particular the acidity of the gastric lumen. The gastric juice in a fasting stomach is approximately pH 2. *H. pylori* is able to resist an environment as low as pH 1 for 3 hours, maintaining an almost neutral cytoplasmic pH [154]. Nevertheless, *H. pylori* first of all avoids the acidic lumen of the stomach and lives close to the epithelial cells. These epithelial cells protect themselves from the gastric acid by the secretion of HCO_3^-, which diffuses only slowly into the lumen, thanks to the gel-like mucus that lines the epithelium. *H. pylori* uses its curved shape and flagella to migrate up the pH gradient into the mucus, attracted by HCO_3^- and urea [112, 117].

Urea, however, is not just a chemo-attractant: it fulfills a key role in the second line of defense against the acidic environment. Urea is hydrolyzed in two NH_3 (ammonia) molecules and one CO_2

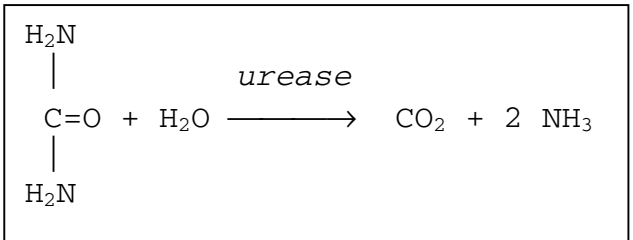

FIG.2: net urea hydrolysis reaction

(carbondioxide) molecule by the enzyme urease (Fig. 2). Under acidic conditions, these ammonia molecules capture H^+ to form NH_4^+, which results in a rise of the pH. The *H. pylori* urease is a heterodimeric enzyme, the subunits are urease A (29.5 kD) and urease B (66 kD) [57, 80]. The importance of this enzyme for *H. pylori* and its protection against acidic conditions can been deduced from the quantities that are produced: 6% of the total protein content of *H. pylori* is urease, [80] an extraordinary large proportion for an enzyme. The *ureA* and *ureB* genes that encode these proteins are in an operon together with a number of accessory proteins [6]. In most bacteria, urease is a strictly cytoplasmic enzyme [114]. In *H. pylori*, urease is also found outside the bacteria, attached to the outer membrane. It has long been believed that in *H. pylori* it functions not only intracellularly, but also extracellularly [132] to veil the bacteria in a 'cloud' of ammonia. The urease was supposed to reach the outside by a process called *altruistic autolysis* [92], a planned cell death of a small portion of the population to provide the others with their extracellular urease. Most evidence, however, indicates that the physiological compartment of *H. pylori* urease is the cytoplasm [146] and that the presence of urease outside the bacteria could be an artifact [139]. A pH-regulated urea channel in the cell wall, encoded by the *ureI* gene, titrates the required amount of urea to keep the internal pH at the desired level. At higher pH values, the channel shuts and thereby decreases the cytoplasmic pH rise when a physiological value is reached [177]. The expression of urease is also downregulated in response to pH rise [7, 170]. Despite this regulation, *H. pylori* will suffer from a lethal pH rise in urea-containing medium if the pH is not buffered below alkaline values [40].

The urease mechanism of acid resistance is well known as a prerequisite for survival, but for active growth at pH 5, which is the pH of the epithelial habitat of *H. pylori*, other mechanisms are also involved [24, 25]. The acid-sensitivity caused by a knockout of these systems is more subtle than of the urease system and their components are at present largely unknown.

Virulence

The outcome of *H. pylori* colonization ranges from asymptomatic gastritis to peptic ulceration, atrophic gastritis and carcinoma. Two important bacterial factors contribute to the virulence of the strain and to the effect of colonization upon the human host: *vacA* and *cagA*.

The *H. pylori* vacuolating toxin, VacA, is a 87 kD protein that causes vacuolation of eukaryotic cells [44, 99]. The *vacA* gene encodes a 140 kD precursor protein [145, 160]. This precursor contains an N-terminal *signal sequence,* which is a short peptide that functions as an

"address tag" for transport across the cytoplasmic membrane, and a C-terminal *autotransporter domain*. This C-terminal domain inserts into the outer membrane and subsequently transports the rest of the protein across the outer membrane, after which the autotransporter domain is cleaved off [145]. At an acidic pH, the secreted mature VacA protein forms hexameric channels in bilayer membranes [45, 86]. VacA is a highly polymorphic protein. Several different phenotypes exist, initially classified as either vacuolating (cytotoxic) or "non-cytotoxic" [13]. The *vacA* gene contains two variable regions: the signal sequence ('S'-region) and a domain in the mature protein ('M'-region). The S regions of different strains can be divided in S1a, S1b, S1c and S2. The S2 allele gives the mature VacA protein a short, hydrophilic extension, which decreases its cytotoxic activity [98]. The M region alleles most often belong to the M1 or the (less abundant) M2 group. The type of *vacA* allele is strongly associated with the presence of peptic ulcer disease, and the cytotoxic effect is presumably the result of the channels they form in cell membranes, but the function of VacA secretion for *H. pylori* remains unclear.

The other pronounced difference in virulence between strains is caused by the *cag*-pathogenicity island (*cag*-PAI), which is present in approximately 60% of *H. pylori* [168]. For unknown reasons, however, the ratio between cag^+ and cag^- strains varies between different parts of the world [42]. In western countries, roughly 40% of the strains are cag^+. In contrast, Thai and Peruvian strains are for 80% cag^+ [131].

The *cag*-PAI was named after the *c*ytotoxin *a*ssociated *g*ene *A* (*cagA*), first recognized as an immunodominant antigen that is present in most cytotoxic strains [41, 166], hence the name "cytotoxin associated gene". This association between *cagA* presence and cytotoxic phenotype was later found to be based on an association between *cagA* presence and *vacA* type S1 [13, 14, 43]. The reason for this association is still not clear. The two genes are located on different chromosomal loci and there is no apparent functional connection. *cagA* is part of a 37kB genomic island, the *cag*-pathogenicity island (*cag*-PAI) that inserts in the glutamate racemase gene (*glr*) between direct repeats of 31 bp [8, 36]. The *cag*-PAI appears to be a dynamic entity. Strains that possess the *cag*-PAI have been shown to contain subpopulations that have lost the *cag*-PAI and have an intact *glr*-gene instead [169]. Thus, the *cag*-PAI appears to be an instable region, which is sometimes deleted from the chromosome.

The *cag* G+C content is 35% [36], considerably lower than the overall G+C content (39%). The *cag*-PAI codes for 27 proteins [60], which form a *type IV secretion system* involved in the direct secretion of the CagA protein into gastric epithelial cells [17, 124, 152] and phagocytic cells [123]. Once inside the host cell, CagA is tyrosine-phosphorylated by host

cell enzymes [16]. Tyrosine-phosphorylated CagA interacts with the tyrosine phosphatase SHP-2 [74], induces dephosphorylation of a number of host proteins [133] and results in cytoskeletal changes, such as long cytoplasmatic extensions and pedestal formation that are known as the "hummingbird" phenotype [147]. The pedestals can be seen in contact with *H. pylori* bacteria. Apoptosis of epithelial cells *in vitro* caused by CagA has been described [96].

Interestingly, *H. pylori* is able to express human blood group antigens Lewis A, B, X, Y, I-antigen, H type 1 and blood group A on its lipopolysaccharide (LPS) [10, 11, 149] that have long been thought to be an exclusive feature of mammals. The expression of these epitopes can be turned on and off by *slipped-strand phase variation, i.e.* 'slipping' of the DNA polymerase on poly(C) tracts in the beginning of a gene, causing changes in the reading frame and therefore on- and off switching of different genes involved in the synthesis of LPS [9, 143]. However, the function of this molecular mimicry is not completely understood. It has been proposed that expression of human blood group antigens helps the microorganism to elude the hosts' immune response and some epitopes appear to serve in attachment to host cells [10, 12].

Epidemiology

In developing countries, *H. pylori* can colonize as much as 90%, while in industrialized countries only 25-50% of the population is colonized [128]. Colonization is mostly established in early childhood [111, 161]. In developed countries colonization rates gradually increase with age due to a birth cohort effect that reflects the socio-economic conditions at younger age for each generation [20, 137].

The mode of transmission of *H. pylori* is not exactly clear, but close contact with a human carrier seems the most important risk factor [167], as was indicated by strain typing experiments using *H. pylori* isolated from children and their parents and siblings [19, 122, 135, 138, 172]. Furthermore, there is also a relation between socio-economic status during childhood and *H. pylori* colonization [176]. On the other hand, evidence for an important extragastric reservoir is lacking, although fecal contamination of water has been implicated in one study [33]. Therefore, most evidence indicates that direct transmission from human to human, either fecal-oral or oral-oral, is the most important route. Especially vomit contains high concentrations of *H. pylori*, which suggests that contact with vomit (directly or indirectly via for example hands or water) could be the mode of infection rather than via saliva or feces [130].

Treatment

Although highly susceptible *in vitro* to a number of commonly used antibiotics, *H. pylori* has proven to be a tough customer when it comes to its eradication from the stomach. The penetration of many antibiotics in the mucus of the stomach is rather low. Furthermore, the acidic pH of the mucus has an adverse effect on the function of many antibiotics. Successful eradication is only achieved at an acceptable rate by a combination of at least two antibiotics with either a gastric acid suppressive drug or ranitidine-bismuth citrate, which combines a moderate antimicrobial effect with gastric acid suppressive activity. The most effective regimens include a proton-pump inhibitor (PPI) with a combination of two of the four commonly used antibiotics: metronidazole, clarithromycin, tetracyclin and amoxicillin [119] for 7 to 10 days. The two regimens most often used as first line therapy are a PPI with clarithromycin and amoxicillin, and a PPI with clarithromycin and metronidazole.

Horizontal gene transfer and natural transformation

Horizontal gene transfer: prokaryotic sex

Usually, bacterial species have a population structure based upon clonal descent (simple cell division). This means that in contrast to sexual reproduction, traits and characteristics are not mixed from generation to generation. In its pure form, this leads to a strictly clonal population structure: every individual of a population is identical to its ancestor unless mutations occur. Mutations that arise in a line of descent will never be present in individuals of another line of descent, unless they arise independently in more than one line of descent. This makes the relationship between individuals of the species very simple and makes it possible to unequivocally determine the order in which the present strains (or individuals) branched off in a phylogenetic tree. With an estimate of the average mutation frequency at this locus (the so-called "molecular clock"), a rough indication of the time that has passed since the last common ancestor lived can be obtained [125].

In most bacteria, however, other mechanisms for genetic recombination exist that allow genetic material to be exchanged between different individuals, albeit not during procreation such as in sexual eukaryotes. This is called "horizontal gene transfer". The word 'horizontal' refers to transfer within one generation, opposed to vertical transfer from ancestor to descendent.

Population structure and genetic variation in *H. pylori*

Soon after the discovery of *H. pylori* it was shown that this species displays a large genetic heterogeneity [106]. The *H. pylori* population structure is almost panmyctic, which means that genetic markers recombine at a frequency that is high enough to eliminate the effect of clonal descent and to generate a linkage equilibrium between alleles at different loci [5, 55, 66, 141, 158]. In other words: new genotypes of *H. pylori* are generated by recombination fast enough to essentially eliminate the effect of clonal descent on its population structure; mutations are recombined too fast to accumulate in a certain clonal lineage. Kersulyte *et al.* identified multiple recombination events between two *H. pylori* strains from the same patient that involve up to 400 bp of DNA [90, 156]. Falush *et al.* investigated genetic relationships of sequential isolates of *H. pylori* from a single patient and estimated that in this set of isolates, the mean size of the recombination fragments was 417 bp and that within 41 years 50% of an *H. pylori* genome is replaced [55]. Clinical isolates obtained from one patient with several years interval, or obtained from members of the same

family show differences when compared with genetic typing methods such as Random Amplified Polymorphic DNA (RAPD), sequencing of DNA fragments, micro-array analysis, and Amplified Fragment Length Polymorfism (AFLP), indicating that genetic alterations occur during prolonged colonization [55, 84, 93, 169]. Recombination has also been demonstrated by RAPD analysis after experimental infection of mice with two *H. pylori* strains [47]. The main mode of horizontal gene transfer used by *H. pylori* is natural transformation.

The ways of DNA: mechanisms of bacterial DNA transfer

The various mechanisms of DNA transfer between prokaryotes can be divided into three main categories: conjugation, transduction and natural transformation. Each has its own characteristics and also a different impact on the population structure.

In the vast majority of reports on DNA transfer events in the natural environment, conjugation is a major mode of horizontal gene transfer [48]. Conjugation is a process defined as a temporary physical connection between two cells, usually both bacteria, during which DNA is duplicated and transferred from one bacterium (the "donor") to the other (the "recipient"). The result is that the recipient ends up with a new, additional sequence of DNA. The DNA that is transferred is not random, but is usually a mobile DNA element: a plasmid or a transposon. This element typically encompasses the system for DNA transfer itself, but can also contain a number of adjacent or integrated genes. Often, these additional genes provide their new host with some optional "accessory functions" that are not essential for normal bacterial growth, but provide the bacterium with a specialized trait that gives a selective advantage under certain circumstances. An appealing example is resistance to broad-spectrum antibiotics [100]. Because most conjugational events only transfer the mobile genetic elements themselves, this type of DNA transfer has relatively little impact on the overall genome structure of bacteria.

The second mode of horizontal gene transfer, transduction, is mediated by bacteriophages (bacterial viruses). Like plasmids, phages can carry specific features for specific circumstances for their hosts. An example is the cholera toxin of phage CTXφ, which turns a harmless sea-dweller into a much-feared human pathogen [171]. The word 'transduction', however, normally refers to bacterial DNA that is transferred by phages. There are two different forms of transduction. The first involves transport of small sequence fragments that are adjacent to the phage integration site and mobilized together with the phage DNA (for example phage λ). The second refers to the erroneous packaging of a random

chromosomal DNA fragment with similar size as the phage DNA into a phage particle. The most well-known example is phage P1 [48]. The resulting "phage" will transfer DNA from the present host instead of phage DNA to a newly infected bacterium.

Natural transformation, the third major mechanism of horizontal DNA transfer, is not initiated by the donor or a mobile element, but by the recipient. It is characterized by active uptake of extracellular DNA over the cell wall of the transforming bacterium. Subsequently, the imported DNA is integrated into the genome by homologous recombination or, if the DNA is a plasmid, by recircularization. Competence, *i.e.* the ability to transform by uptake of extracellular DNA, requires a dedicated DNA uptake system, which is present in some bacterial species only. Intriguingly, many mucosal pathogens are competent: *Neisseria* spp., *Haemophilus* spp., *Streptococcus* spp. (including *S. pneumoniae*) and the subject of this thesis: *H. pylori*.

DNA uptake by transformation is not limited to specific DNA elements, and therefore natural transformation is the DNA exchange mechanism with the strongest impact on the population structure of the species. The only prerequisite is that (part of) the incoming DNA contains enough homology to be integrated in the chromosome by homologous recombination. The consequence of natural transformation is that competent bacteria tend to recombine random genomic sequences, which seriously disrupts their clonal population structure [79].

Intermezzo: natural transformation and the discovery of DNA

The discovery of natural transformation, and subsequently the discovery that DNA is the carrier of hereditary traits, came from an unexpected observation during studies on pneumococcal virulence by Fred Griffith. In the 1920's, Griffith was experimenting with "smooth" (encapsulated) and "rough" (unencapsulated) pneumococci. The polysaccharide capsule that causes the colony to become smooth was not yet discovered, but Griffith noticed that when he mixed living, rough pneumococci with killed smooth pneumococci, he obtained pneumococci that had "transformed" to the smooth phenotype! He was forced to conclude that the dead, smooth bacteria could transfer their smoothness to living rough pneumococci and that this trait would then pass on to their progeny. He called this process *transformation* and the unknown substance that transferred the smoothness the "transforming principle".

At that time, the substance that was responsible for heredity was not yet known, and Griffith had found the key to its discovery. But it was not obvious to Griffith how interesting his "transforming principle" actually was. He does not mention the words gene, genome or heredity anywhere in his publication on the transforming principle [71].

FIG. 3: Oswald Avery in 1941 [105]

Three other investigators, Oswald Avery (Fig. 3), Colin McCleod en Maclyn McCarty, continued the search for the identity of the transforming principle. Like Griffith, they worked in the field of pneumococcal disease rather than genetics, but they gradually came to realize that Griffith's "transformation" was caused by transfer of heritable traits and that the "transforming principle" must be the carrier of heredity [105]. It took them 10 years to prove that the carrier of genes is, in fact, DNA [15]. At the time, their work received a half-hearted (if not unbelieving) reception [105]. Probably, during the Second World War people had other things on their minds than reaction vessels with invisible chemical substances. By the time the famous Watson and Crick built their photogenic model of a double helix, the three discoverers of DNA had largely been forgotten.

Natural transformation: introduction

The process of natural transformation can be divided in four steps: development of a competent (uptake-proficient) state, binding of the extracellular DNA, transport of the DNA across the cell wall, and integration into the genome. The last step usually implies either reconstitution of a plasmid, or RecA-dependent homologous recombination with the bacterial chromosome [37, 153].

The first step, development of a competent state, is highly dependent on the species involved. Gram-positive species, such as *Bacillus subtilis* and streptococci [39, 53, 101, 163] tend to develop competence in a certain phase of their cell cycle, under control of quorum sensing pheromones. Gram-negative species have not been found to regulate competence with quorum-sensing, but other forms of regulation are present. In *Neisseria gonorrhoeae* the development of competence is dependent on the expression of type IV pili [3, 27]. In

Neisseria meningitidis competence is constitutively expressed. *Campylobacter jejuni* and *H. pylori* are most competent in early log phase [18, 83, 173]. When the bacteria are in a competent state, transformation can take place. Most data about this process are available in *B. subtilis* and, to a lesser extent, *H. influenzae, S. pneumoniae* and *N. gonorrhoeae*. These data will be summarized below.

When bacteria have reached their competent state, transformation starts with binding of extracellular DNA. A receptor has been identified with homologs in Gram-positive (*B. subtilis* [82], and *S. pneumoniae* [23]) and Gram-negative (*N. gonorrhoeae* [38]) species, but not in *H. pylori*. After cleavage by an extracellular endonuclease, one strand of the DNA is degraded while the other is taken up over the cell wall. Gram-positive micro-organisms subsequently take up DNA indiscriminately. Some Gram-negative microorganisms, however, recognize DNA by an 'uptake sequence' of approximately 10 bp that is highly abundant in their own species [46, 70, 174]. DNA from other species remains unbound and will not be taken up.

The process of uptake itself remains rather mysterious. In *N. gonorrhoeae* the binding and uptake of DNA are dependent on the expression of proteins involved in type IV pilus assembly as well as on structural pilus subunit proteins, which suggests that the pilus itself facilitates the DNA uptake in some way. However, recent data show that the uptake is not dependent on intact pili [2, 73, 103]. These seemingly contradictory data could be explained by short "pseudopili", assembled from the same subunits as the real pili that protrude from the surface of the bacterium. Likewise, other competent organisms have an uptake system that shares homology with type IV pili and appear to form a similar DNA binding pilus [37]. The only known exception is *H. pylori*. Rather than resembling pili, the structural core of the *H. pylori* DNA translocation apparatus is related to the type IV secretion system [78].

Type IV secretion and *H. pylori* transformation

Type IV secretion systems translocate bacterial macromolecules to the extracellular compartment or directly into target cells. The translocated molecules range from proteins that are injected into the mammalian host cells to plasmid DNA transferred during conjugation. Although the particular set of protein components varies between type IV systems, they generally comprise a pilus-like mating system and a mating channel that spans the bacterial cell wall. The archetypal type IV secretion system is *vir*, encoded by the *Agrobacterium tumefaciens* Ti-plasmid. Ti stands for "Tumor inducing", describing the neoplastic root nodules that are typical of crown gall, a plant disease caused by *A. tumefaciens.* These

nodules are induced upon the introduction and expression of a DNA segment of the Ti-plasmid, called T-DNA, into plant cells by the *vir* system. This T-DNA is subsequently transported to the plant nucleus, andd integrated in the plant genome. There it directs the production of both plant-tumor inducing phytohormones and novel plant metabolites known as opines. *A. tumefaciens* strains containing the Ti-plasmid are able to utilize these novel metabolites as a source ofr energy and carbon. The total set of proteins involved in *vir*-mediated DNA transfer includes the *virB* operon that encodes VirB1 through VirB11, and VirD4. The transmembrane transport pore is proposed to consist of four proteins: VirB7, VirB8, VirB9 and VirB10 (Fig. 4).

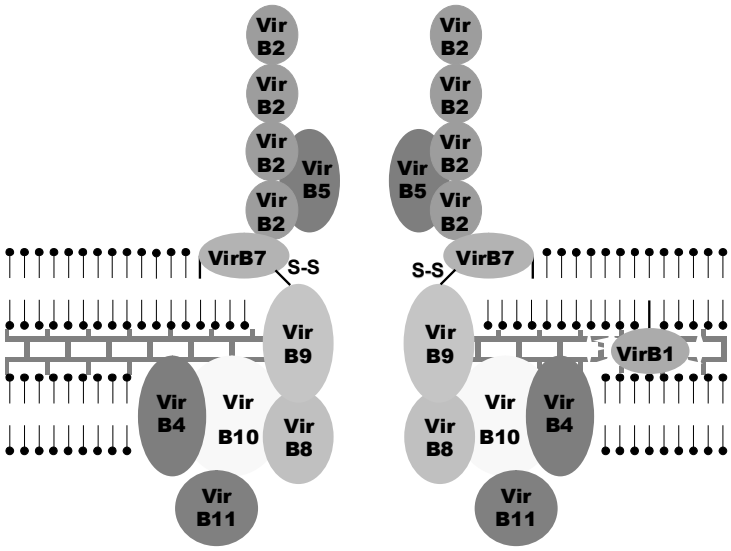

FIG. 4: schematic outline of *vir*, the archetypal type IV secretion system

The *H. pylori comB* transformation system as described by Hofreuter *et al.* [77] and Karnholz *et al*. [89] carries homologues of core components of the type IV secretion system, *i.e. comB2, comB3, comB4, comB6*, *comB7*, *comB8*, *comB9* and *comB10* (the latter were formerly designated *orf2*, *comB1*, *comB2* and *comB3*). The *comB* genes have the same co-linear organization as their *virB* counterparts with equivalent numbers and, perhaps more importantly, the predicted cellular topology of the ComB protein products strongly suggests that they resemble the building blocks of the VirB transport pore at a structural level (Fig. 5).

The four genes *comB7, comB8, comB9* and *comB10* are organized in a tandem arrangement with small overlap in their reading frames [77]. The first reading frame of the operon, *comB7*, is predicted to code for a peptide with a putative lipoprotein signal sequence [76]. VirB7 binds VirB9 through disulfide bonding between two cysteine residues [67] and this bond is necessary for the assembly of a stable transport apparatus [97]. The cysteine residues involved in this bonding are conserved in ComB7 and ComB9. Moreover, ComB9 and ComB10, when overproduced in *H. pylori*, are degraded faster in the absence of ComB7 and ComB8, an indication of a functional analogy between VirB7 and ComB7 [77]. ComB8

is, like VirB8, a membrane-anchored protein with a large periplasmic domain [76]. ComB9 and ComB10 are also located in the periplasm, but like in the Vir system, only ComB10 remains anchored in the cytoplasmic membrane [76, 77]. Together, the ComB proteins have all the characteristics of a pore-forming transmembrane complex, suitable for the translocation of DNA over the cell envelope.

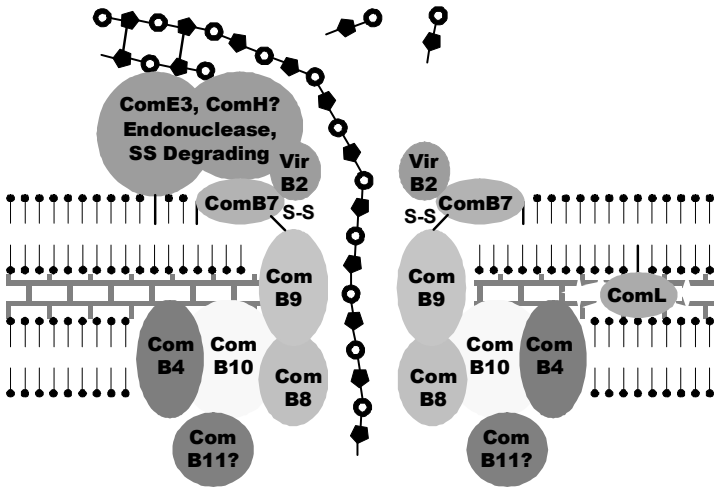

FIG. 5: schematic outline of a putative *H. pylori* DNA uptake system. For details see text.

The energy for the molecular transfer through type IV systems is provided by homologs of the membrane bound ATPasesVirD4, VirB4 and VirB11. *H. pylori* contains three chromosomal VirB4 homologs (HP0017, HP0441 and HP0459), one VirB11 (HP1421) and a VirD4 (HP1006) homolog [164]. In *A. tumefaciens,* VirD4 is thought to be involved in the coupling of the T-DNA-protein complex to the transport system [75], and such a function is probably dispensable in the reversed transport direction of DNA in transformation.

VirB4 and VirB11 both have a proposed function in the transport system itself. The VirB4-homolog encoded by HP0017, 23 kb upstream of the *comB* locus, interacts with ComB10 [134]. Hofreuter *et al.* have shown that a null mutant in this protein is indeed affected in its transformation [77] and designated this *virB4* homolog *comB4*. VirB2, VirB3, and VirB6 serve as structural components of the *A. tumefaciens* T-pilus (VirB2) [118, 144] or are involved in its assembly (VirB3, VirB6) [72]. Pili have never been described in *H. pylori*. ComB2 might be part of a pseudopilus in the cell wall that consists of pilin subunits that do not assemble to form a true pilus [89] similar to the proposed pseudopili in *N. gonorrhoeae*.

Vir proteins not represented in the ComB system: VirB1, VirB5 and VirB11

Three Vir proteins have no counterpart in the *H. pylori* transformation system. The first is VirB1. VirB1 is targeted to the periplasm and there spliced in a C-terminal and a N-

terminal half, which both have a different function [21]. The N-terminal subunit remains in the periplasm and is involved in murein hydrolysis to provide the channel for the Vir-transport system, the C-terminal subunit is secreted. Its function is not exactly clear [102]. In *H. pylori*, the murein hydrolysis necessary for the assembly of a DNA transport channel could be performed by ComL, a homolog of a murein hydrolase of *Neisseria gonorrhoeae* involved in natural transformation [63].

VirB5 is a structural component of the *A. tumefaciens* T-pilus [118, 144]. Its absence could be explained by the absence of a true pilus in the *H. pylori* transformation system, which makes this protein unneccessary. VirB11 interacts with VirB9 and VirB10 [181] and is involved in the assembly of the transport system. Although *H. pylori* contains a *virB11* homolog, this gene is not involved in natural transformation [89].

Other components of the *H. pylori* transformation system

The differences between import and export create different demands for the type IV-like DNA uptake system than for the type IV secretion systems. The first step in natural transformation is the binding of the extracellular double-stranded DNA. Next, transforming species usually restrict the DNA to fragments of suitable length before transport into the cytoplasm. The imported DNA is usually single stranded [73] just as the exported DNA in type VI systems. These requirements indicate that at least a DNA binding function and probably also endonuclease and single strand-degrading functions are required in the *comB* system. It is tempting to speculate that these tasks are fulfilled by one or more proteins that interact with ComB7, in analogy with the T-pilus association with VirB7 in *A. tumefaciens* [140]. The HP1361 protein, a homolog of the *B. subtilis* ComE3, is probably involved in an early step of DNA translocation because it is not only essential for transformation, but also for DNA binding [180]. With the analogy to type IV secretion systems in mind, an outline of a minimal *H. pylori* DNA uptake system is represented in figure 5.

After the uptake: integration in the genome

After the DNA is imported into the cytoplasm, it will be degraded unless swift action is taken. The kind of action that is taken differs between plasmids and other DNA fragments. Chromosomal DNA fragments need to be recombined into the chromosome (or a plasmid) by recombination. This depends strongly on the presence of near-complete homology between the new DNA and the host DNA. In *E. coli* a homologous sequence of at least 23 bp is necessary for the RecBCD pathway and of 44 bp for the RecF pathway. If the homology

drops from 100% to 90%, recombination efficiency decreases dramatically [148]. This is, apart from the requirement of an uptake sequence in some Gram-negative species, the most important barrier against the uptake of DNA from unrelated species. No transformation with chromosomal fragments can be shown in recombination-deficient strains.

The available evidence suggests that plasmids, similar to chromosomal DNA, are taken up in linear form in *N. gonorrhoeae* [26] and *B. subtilis* [50, 95]. This implies that plasmids need to be recircularized in the cytoplasm. This could be explained by the uptake of at least a single-stranded fragment of both strands of the plasmid, such that one fragment can glue both ends of the other fragment together. This hypothesis is supported by the fact that the presence of a homologous stretch of DNA in the chromosome or a residing plasmid enhances the frequency of plasmid uptake in *B. subtilis* [34] *S. pneumoniae* [104, 150] and *H. influenzae* [155]. Interestingly, plasmid transformation without homology does occur in recombination-deficient recipients of these species. Homology with chromosomal sequences or resident plasmids can enhance plasmid transformation, but this effect of the homology is observed only in Rec^+ strains.

Scope of this thesis

The magnitude of natural transformation is mainly deduced from phylogenetic data. To find detailed footprints of natural transformation, *H. pylori* strains are examined for traces of horizontal DNA transfer *in vivo* (chapter two). In chapter three, three *H. pylori* homologs of known transformation genes are tested for a function in transformation. The function of one of these genes, *dprA*, is studied in more detail in chapter four. In chapter five, a search for elements of the *H. pylori* transformation system without previous knowledge of other transformation genes from other species is described. This search resulted in the identification of a novel transformation factor, called ComH. In chapter six, the effect of restriction-modification systems on the genomic sequence of two *H. pylori* strains is investigated.

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Chapter 2:

Molecular patchwork: chromosomal recombination between two *Helicobacter pylori* strains during natural colonization

Smeets LC, Arents NL, van Zwet AA, Vandenbroucke-Grauls CM, Verboom T, Bitter W, Kusters JG. (2003) Infect Immun. 71(5):2907-2910

Abstract

Genetic analysis of two *Helicobacter pylori* strains isolated from a single gastric biopsy showed evidence of extensive horizontal gene transfer. Several large recombinations were identified in the *rdxA* gene, which is involved in metronidazole resistance.

The gastric pathogen *Helicobacter pylori* displays considerable genetic diversity (2). Clinical isolates obtained from one patient with several years interval show evidence of genetic drift during prolonged colonization (7;10;13). The existence of isogenic *cag*-positive (*cag*+) and *cag*-negative (*cag*-) strains indicates that the *cag* pathogenicity island (*cag*-PAI) can be lost from the chromosome (19). Several studies have demonstrated that recombination occurs frequently enough to virtually eliminate the effect of clonal descent on the population structure of *H. pylori* and to generate a linkage equilibrium between alleles at different loci (1;8;15;17). This variability can be significant for the outcome of *H. pylori* infection. For instance, the presence of the *cag*-PAI and allelic variation in the vacA and iceA genes are associated with development of peptic ulceration and gastric cancer (4;21).

In *H. pylori*, random mutations that inactivate *rdxA* cause metronidazole resistance (9). In a previous study, we reported on biopsy specimens that contain both metronidazole-sensitive (Mtz-S) and -resistant (Mtz-R) colonies (3). Genotyping by RAPD and by RFLP of the ureC gene showed that in one of the biopsies, biopsy BH9809-109, two different strains are present. Interestingly, both strains contain Mtz-S as well as Mtz-R isolates. In the present study we determined whether horizontal gene transfer has occurred between these two strains. In the *rdxA*-locus extensive recombination was observed.

Strain identification. Twelve isolates were subcultured from biopsy BH9809-109 and named L1 – L12 according to increasing Mtz resistance (Table 1). Seven isolates were Mtz-S (L1 – L7, MIC < 8 mg/l, table 1) and five were Mtz-R (L8 – L12, MIC ≥ 8 mg/l). Six Mtz-S isolates (L1 – L4, L6 and L8) and three Mtz-R (L9, L11 and L12) belonged to the first genotype (strain 1). The second genotype (strain 2) included two Mtz-S isolates (L5 an L7) and one Mtz-R isolate (L10). *cag*- and *vacA*-status were assessed by Line Probe Assay (LiPA) (20). Strain 1 contained *vacA* type s1a/m2 and was *cag*+, strain 2 had *vacA* type s2/m2 and was *cag*- (Table 1). Neither strain showed variation in *vacA* or *cagA* status among individual colonies; this indicated that no recombination affecting the *cag*-PAI or vacA had taken place.

The rdxA alleles. Because both strains contained Mtz-S as well as Mtz-R isolates and reversion of nonsense mutations is unlikely, resistant subpopulations appeared during colonization from the Mtz-S ancestors of either strain (with intact *rdxA* alleles). To demonstrate a possible transfer of a resistant *rdxA* allele from one strain to the other, we sequenced the *rdxA* gene in all twelve isolates. The results are shown in figure 1. Two different alleles of the *rdxA* gene (allele A and allele B) were present in the set of strains, which differed at 22 base positions in the 630bp ORF. However, the distribution of the *rdxA*

TABLE 1. Metronidazole susceptibilities, genotypes, *rdxA* alleles, presence of the *cag* PAI, and *vacA* type of isolates L1 to L12

Isolate	Metronidazole MIC (mg/liter)[a]	Genotype[b]	*rdxA* allele	*cagA* gene present	VacA type
L1	0.5	1	B	+	S1a/M2
L2	0.5	1	B	+	S1a/M2
L3	0.38	1	A	+	S1a/M2
L4	0.75	1	A	+	S1a/M2
L5	1.5	2	A	-	S2 /M2
L6	1.5	1	A	+	S1a/M2
L7	1.5	2	A/B	-	S2 /M2
L8	48	1	A	+	S1a/M2
L9	>256	1	A	+	S1a/M2
L10	>256	2	A	-	S2 /M2
L11	>256	1	A	+	S1a/M2
L12	>256	1	A	+	S1a/M2

[a] Sensitive, <8 mg/liter; resistant, ≥ 8 mg/liter.
[b] According to random amplified polymorphic DNA typing and restriction fragment length polymorphism of the ureC gene.

alleles did not correspond to the genotypes of the isolates. Strain 1 contained isolates with *rdxA* allele A (L3, L4, L6, L8, L9, L11 and L12) as well as isolates with *rdxA* allele B (L1 and L2). Of the isolates from strain 2, L10 had allele A but the other two isolates of strain 2 (L5 and L7) possessed a mixed allele. L5 had an allele that was predominantly type A, with a single polymorphism at base position 549 consistent with allele B. This might indicate transfer of a small DNA fragment, but could also result from a mutation that happened at this polymorphic locus. In L7, the three polymorphic sites at the 5'end region of the ORF were identical to the allele B group, whereas the other 19 polymorphisms in the middle and at the 3'end were concordant with the allele A group. Between the allele A and allele B regions of L7, there was a gene fragment of 77 bases with perfect homology between allele A and allele B. This indicates that a recombination event introduced part of the *rdxA* locus of strain 1 into the L7 genome.

Origin of the metronidazole resistance. All resistant isolates contained *rdxA* allele A, which suggests transfer of the resistance mutation. However, there was a difference between the resistant isolate of strain 2 (L10) and the resistant isolates of strain 1 (L8, L9, L11 and L12). In L10, a single basepair deletion was found at base position 192 of the ORF, causing a frameshift that interrupts the reading frame. This frameshift occurred in a homopolymeric stretch of 10 adenine residues interrupted by one cytosine. An identical deletion was described earlier in an unrelated strain (11). Apparently, this locus is vulnerable to slipped

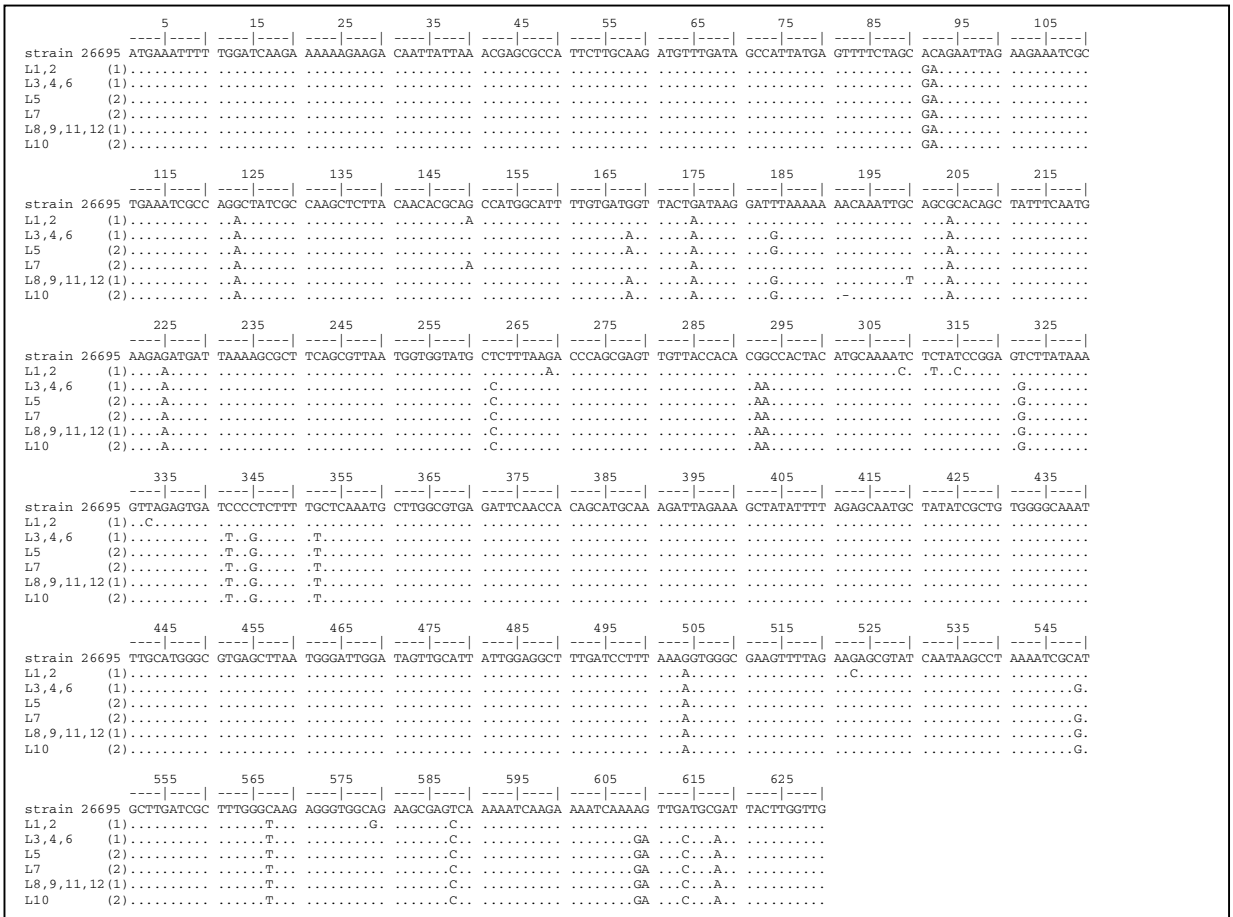

FIG. 1. Alignment of the *rdxA* ORFs of isolates L1 – L12 with reference strain 26695. Between brackets the strain type (1 or 2).

strand mispairing. The resistant isolates of strain 1 (L8, L9, L11 and L12) did not possess this frameshift but showed a C to T mutation at position 200 of the ORF, which causes an amino-acid change from alanine to valine. To confirm that this minor difference causes metronidazole resistance, we used a PCR amplimer of the L8 *rdxA* allele for natural transformation (22) to susceptible *H. pylori* strains. This yielded MtzR transformants with a frequency of 10-3 per recipient cell, which indicated that this mutation is sufficient to cause metronidazole resistance. We conclude that resistance in both strains arose by an independent mutation of the *rdxA* gene after transfer of an intact *RdxA* allele A had taken place. Despite the high frequency of horizontal gene transfer between strain 1 and strain 2, a *rdxA* mutation arose independently in both strains.

The region around rdxA. In order to determine the length of the recombinations between strain1 and strain 2, the DNA sequence on both sides of the *rdxA* ORF was determined for two isolates from strain 1 (L1 with allele B and L4 with allele A), as well as two isolates of strain 2 (L7 with 5' allele A / 3' allele B and L10 with allele A). The results

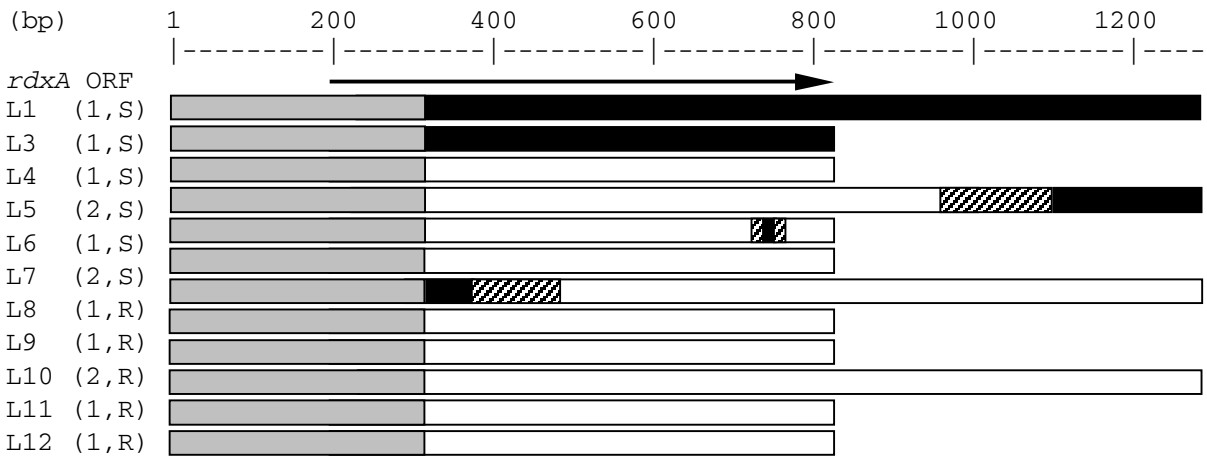

FIG. 2. Distribution of alleles A and B in isolates L1 to L12. Numbers in parentheses indicate the genotype (1 or 2). S and R, metronidazole susceptibility and resistance, respectively. The arrow shows the *rdxA* ORF. Open bars, allele A; solid bars, allele B; hatched bars, crossover sequence; gray bars, identical sequence. Strain L4 and strain L7 contain recombined sequences. The point mutations that cause resistance in the resistant isolates L8 to L12 are not shown.

are shown in figure 2. Downstream, another crossover between the two alleles was identified: after a perfect homology of 145 bp between the four sequences, the sequence of the strain 1 isolates L4 and L1 were identical over a length of at least 205 bp with 8 mismatches between strain 1 (L1/L4) and strain 2 (L7/L10). This is concordant with an initial combination of strain 1 with allele B and strain 2 with allele A. Upstream of the *rdxA* ORF, however, the sequences of L1, L4, L7 and L10 were identical over a length of >1,7Kbp (results not shown). It is unlikely that the original sequences of strain 1 and 2 were already identical before their co-habitation, because in this region there is only 94% conservation between these strains and the published genome sequences (2;18). Apparently, a previous, major recombination event had replaced the original sequence in one of the strains, which results in an identical sequence in all four isolates. In an attempt to identify the other original sequence, from the remaining eight sequences a fragment of 800 bp upstream of *rdxA* was sequenced but found to be identical to the others.

Most observations on natural horizontal gene transfers concern transfer of genetic elements that are mobile by nature, such as plasmids or transposons. Horizontal gene transfer of non-mobile elements is only rarely observed outside experimental settings (14). It is usually inferred from sequence analysis of unrelated isolates of mucosal pathogens that are competent for DNA exchange by natural transformation: Streptococcus pneumoniae, Neisseria spp., *Haemophilus influenzae* and *H. pylori*. In some cases, the recombining sequences must have been over 1 kb long (5;6). For *H. pylori*, its isolated habitat in the gastric

mucosa with prolonged contact between different strains provides the unique opportunity to isolate recombining strains together. Kersulyte *et al*. identified recombination events between two *H. pylori* strains from the same patient that involve up to 400 bp (12). Falush *et al*. investigated genetic relationships of sequential isolates of *H. pylori* from a single patient and estimated the mean size of the recombination fragments to be 417 bp (7).

Here, we demonstrate that multiple homologous recombination events can add up in a single area of the chromosome. The sequences upstream of the *rdxA* ORF are identical for at least 1.7 kb, which suggests that a large fragment was transferred and integrated in the ancestors of isolates L1 – L12 and that *H. pylori* can exchange DNA fragments long enough to contain one or more complete genes. The lack of polymorphic sites for over 1.7 kb obscures two cross-over points in this area and the length of two other recombining sequences of at least 600 bp in L4 and of at least 25 bp in L7 (Fig. 2). Two cross-overs had taken place at 145 and 77 bases of perfect homology, respectively. This is in accordance with experimental data, which indicate that recombination efficiency is highly dependent on a perfect homology of sufficient length (16). The molecular patchwork that is described here illustrates the frequent recombination during co-colonization of *H. pylori* strains that leads to the panmyctic population structure of this species.

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Chapter 3:

The *dprA* gene is required for natural transformation of *Helicobacter pylori*

Smeets LC, Bijlsma JJ, Kuipers EJ, Vandenbroucke-Grauls CM, Kusters JG. (2000) FEMS Immunol Med Microbiol. 27(2):99-102

Abstract

Genetic recombination in *Helicobacter pylori* is believed to be involved in host adaptation of this gastric pathogen and uptake of DNA by natural transformation can result in changes in virulence factors as well as antigenic variation. To elucidate the mechanisms involved in natural transformation we tested two genes with homology to known competence genes (*dprA* and *traG*) for their role in this process. Insertion mutants in these genes were constructed in two different *H. pylori* strains and their competence by natural transformation was compared to the wild-type. Mutation of the *traG* homolog did not reduce competence. Mutation of the *dprA* gene, however, severely impaired natural transformation both with plasmid and chromosomal DNA. Our data indicate that *dprA* and *comB3* are essential parts of a common pathway for chromosomal and plasmid transformation.

Introduction

Helicobacter pylori is a Gram-negative bacterium that colonizes the human stomach and causes chronic gastritis, peptic ulceration and is associated with the development of gastric neoplasms. Recombination between *H. pylori* strains is extremely common [1] and novel subtypes appear during colonization [2,3]. More than half of the *H. pylori* strains contain a pathogenicity island, the *cag* region, whose presence has a marked influence on the virulence of the organism. This *cag* region was probably acquired by DNA uptake from a di¡erent species [4]. Colonization with two or more strains is common [5] and during this co-colonization extensive genetic recombination takes place, including changes in important virulence markers such as the *cag* status [3]. These facts indicate that uptake of foreign DNA contributes to virulence and host adaptation of *H. pylori*. *H. pylori* is naturally competent for transformation [6], a process whereby a bacterium takes up exogenous DNA and incorporates it into its own genome. This is probably an important mode of DNA transfer for *H. pylori*, albeit other mechanisms like conjugation [7] are possibly involved as well.

Despite its natural competence, no integral DNA uptake system could be identified in the published genomic sequences of *H. pylori* [8,9]. Based upon homology with genes involved in DNA-exchange mechanisms in other species, Tomb *et al*. proposed that a number of open reading frames are involved in transformation [9]. These open reading frames have homologs in a wide variety of species and they do not form a distinct locus or operon, which raised the question whether all of these genes indeed function in the *H. pylori* competence mechanism. Hofreuter *et al*. have recently shown that the *comB* locus is involved in competence [10]. In this study we sought to test the proposed function of two homologs of competence genes: *dprA*, a homolog of a transformation gene in *Haemophilus*, and *comL*, a homolog of a transformation gene in *Neisseria*. A *traG* homolog was also included in this study. Like *comB3, traG* has homologs which are involved in conjugational DNA transport rather than transformation. Only in *dprA* and *traG H. pylori* mutants could be obtained. The competence of the mutants was compared with the parental strain and a *comB3* mutant by natural transformation with both chromosomal DNA and plasmids. Because of the large genetic divergenge in the genus *H. pylori*, mutants were produced both in the moderately competent reference strain NCTC11637 and in the highly competent clinical isolate 1061.

Materials and methods

Bacterial strains and culture conditions. H. pylori strains NCTC11637 and 1061 [11] were grown under microaerobic conditions on Columbia agar supplemented with 7% lysed

horse blood, Dent's *H. pylori* selective supplement (Oxoid, UK) and 40 mg/l of 2,3,5-triphenyltetrazolium chloride (Sigma Co., USA). referred to as Dent-plates. When appropriate, one of the following antibiotics was added: kanamycin 10 mg/1 (Sigma), chloramphenicol 10 mg/l (Serva, Germany) or clarithromycin 2 mg/l (Abbott laboratories ltd., UK). Plasmid pHel2 is an *Escherichia coli-H. pylori* shuttle vector [12] that carries the Cat_{GC} chloramphenicol resistance gene. Vector pBCα3 [11] contains a kanamycin resistance cassette (*aphA-3*) and an *E. coli* chloramphenicol resistance marker (CamR).

Construction and competence assessment of mutants in H. pylori. DNA manipulations were performed according to standard protocols [13]. To construct pBCα3-derived suicide vectors a 300 bp fragment from the middle of *comB3, dprA* and *traG* was amplied by PCR and ligated into pBCα3. *H. pylori* mutants were created by natural transformation with these suicide constructs. Mutants were obtained at a frequency of approximately 100 colonies and 1 colony per μg vector for strain 1061 and NCTC11637, respectively. Disruption of the correct gene was confirmed by plasmid rescue as described earlier [11]. Sequence analysis of the rescue plasmids showed that the vector was inserted at the expected site. We determined the competence of the mutants by natural transformation as described by Wang *et al*. [14], with 1 μg of chromosomal DNA isolated from a clarithromycin-resistant mutant of strain NCTC11637 (MIC > 32mg/l) [15] or 500 ng of plasmid pHel2 isolated from *E. coli* strain ER1793 (New England Biolabs, USA). After 24 h the bacteria were harvested and suspended in PBS. 100 μl of this suspension as well as 1:10 and 1:100 dilutions were spread on Dent plates containing clarithromycin or chloramphenicol and the number of resistant colonies was determined. To determine the number of viable cells, dilutions of $1:10^6$, $1:10^7$ and $1:10^8$ were plated on Dent-plates. The transformation frequency was defined as the number of resistant colonies divided by the total number of viable bacteria.

Results

Transformation of the mutants with chromosomal DNA. Two independent mutants in each gene were selected and compared to the wild-type in three separate transformation experiments. Although the frequencies tend to vary from experiment to experiment, in the wild-type strain NCTC11637 typically one transformant per 10^6 viable cells was found using 1 μg of homologous DNA. Within a single experiment, the transformation frequency of strain 1061 is 50 times higher than the frequency of NCTC11637. The transformation daa4 obtained for our mutants are summarized in Table 1. To relate the mutants to the wild-type, the frequencies are depicted as a percentage of the corresponding wild-type's transformation

frequency conform the method of Hofreuter *et al*. [10]. As can be seen from the table, *comB* mutants did not transform at all. Mutation of the *dprA* gene strongly affected transformation, this effect was most pronounced in strain 1061 (Table 1). The competence of *traG* mutants was equal to the wildtype frequency.

TABLE 1: transformation frequency of the mutants with 1 µg of chromosomal DNA or 500 ng of plasmid pHel2.

Gene	Strain NCTC11637		Strain 1061	
	Chromosomal DNA	Plasmid pHel2	Chromosomal DNA	Plasmid pHel2
Wild type	$5*10^{-7}$ (100%)	$8*10^{-6}$ (100%)	$2*10^{-5}$ (100%)	$3*10^{-5}$ (100%)
comB3 HP0043	0 (<0.01%)	0 (<0.01%)	0 (<0.01%)	0 (<0.01%)
dprA HP0333	$3*10^{-9}$ (0.6%)	$2*10^{-8}$ (0.25%)	0 (<0.01%)	$1*10^{-8}$ (0.03%)
traG HP1006	$6*10^{-7}$ (120%)	-	$2*10^{-5}$ (100%)	-

Transformation frequencies in a representative experiment, between brackets the relative transformation frequency of the mutants as a percentage of the wild-type.

Transformation of the mutants with plasmids. While transformation of chromosomal DNA depends on allelic exchange through recombination, transformation with plasmids most often occurs independent of recombination [16,17]. To investigate whether the *comB* and *dprA* genes also play a role in transformation with a *H. pylori* plasmid, the mutants were also tested for transformation with the self-replicating plasmid pHel2 (Table 1). The wild-type NCTC11637 typically transforms at a frequency of 6 per 10^6 viable cells per 500 ng of plasmid and strain 1061 at 40 per 10^6. A plasmid of the expected size was present in a random sample of transformants from our experiments, which indicates that pHel2 is maintained as a plasmid and the obtained chloramphenicol resistance was not due to Cat_{GC} marker rescue. For *comB3* and *dprA* mutants no major differences were observed between transformation with either chromosomal DNA or plasmids as the DNA source.

Discussion

Horizontal gene transfer in *H. pylori* has gained increased attention because it appears to be common in this species [1,3] and natural transformation is assumed to play an important role in this process. On the biological relevance of competence, however, one can only speculate. It might be involved in the transfer of virulence factors and antibiotic resistance, and facilitate antigenic variation by recombination.

In our study, only for *dprA, traG* and *comB3* mutants were obtained. No *H. pylori* mutants in the *comL* gene could be made upon repeated attempts, which might indicate that *comL* disruption is lethal in *H. pylori*. It was therefore decided to concentrate on *dprA* and *traG*.

In both strains, disruption of the *comB3* gene resulted in the complete loss of competence for transformation with a chromosomal marker. This is in agreement with experiments by Hofreuter *et al.* [10]. We also tested *comB3* mutants for their ability to take up self-replicating plasmids and found that transformation is equally impaired. From this we conclude that *comB3* is not involved in chromosomal integration but in a previous stage of the transformation process, *e.g.* uptake of DNA.

The *traG* gene was named after a conjugation gene located on the *Agrobacterium tumefaciens* Ti-plasmid. No significant decrease in transformability was found for the *H. pylori traG* mutants. Since *H. pylori* is capable of conjugation [7], this gene, although located on the chromosome, might be involved in conjugation.

dprA mutants showed a significant decrease of transformation capacity. Complementation of the gene by the introduction of an intact copy was hampered by the mutants' lack of competence. *H. pylori dprA* belongs to a gene family with members in different species. Not all of those species are known to be naturally competent, but a mutation in the *Haemophilus influenzae dprA*. reduced transformation 10,000-fold compared to the wild-type [18]. In contrast to its *H. pylori* homolog, however, the *H. influenzae* mutant is capable of transformation at a wild-type level if plasmids are used as a DNA source. Karudapuram *et al*. suggested that different types of processing for linear DNA and plasmids during transformation of *Haemophilus* could explain this difference [19]. If that is correct, the processing of plasmids in *H. pylori* must be different from the processing in *Haemophilus*. Alternatively, *dprA* could be a recombination gene both in *Haemophilus* and in *H. pylori*, because it has been shown that plasmid transformation in *H. pylori* is not strictly independent from RecA function [12].

In conclusion, both *dprA* and comB3 appear to be part of a common pathway for chromosomal and plasmid transformation. Further research is necessary to establish the role of *dprA* in more detail.

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Chapter 4:

Functional characterization of the competence protein DprA/Smf in *Escherichia coli*

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Abstract

In several bacterial species that show natural transformation, *dprA* has been described as a competence gene. The DprA protein has been suggested to be involved in the protection of incoming DNA. However, members of the *dprA* gene family (also called *smf*) can be detected in virtually all bacterial species, which suggests that their gene products have a more general function. We examined the function of the DprA/Smf homologue of *E. coli*. *E. coli dprA*/*smf* is able to partially restore transformation in a Haemophilus influenzae *dprA* mutant, which shows that *dprA*/*smf* genes from competent and non-competent species are interchangeable with respect to their involvement in natural transformation. From this we conclude that natural transformation is probably an additional function of these genes. Subsequently, the *dprA*/*smf* gene was deleted in various recombination mutants of *E. coli* and the resultant phenotype was tested. All the resultant *E. coli dprA*/*smf* mutants did not differ from their parent strains with respect to transformation, Hfr-conjugation, recombination and DNA repair. Therefore, a role of DprA/Smf in DNA recombination could not be established and the basic function of *dprA*/*smf* remains unclear.

Introduction

Naturally competent bacteria have the ability to actively take up extracellular DNA and to integrate it in their genomes. This process is called natural transformation. The donor DNA can be chromosomal, phage or plasmid. Chromosomal fragments have to recombine with the chromosome after uptake, whereas plasmids and phage may need to be recircularized. Naturally competent bacteria have a dedicated system for the binding and uptake of extracellular DNA. The components of these systems are similar in the different species, with the exception of *Helicobacter pylori* (Chen *et al*., 2005). Subsequent integration of chromosomal fragments depends on recombination. RecA, present in both transformable and non-competent species, plays a central role in homologous recombination. DprA is also present in most bacterial species, but its function is more obscure. DprA has been identified as a transformation protein in *Haemophilus influenzae* (Karudapuram *et al*., 1995), *H. pylori* (Ando *et al*., 1999;Smeets *et al*., 2000), *Thermus thermophilus* (Friedrich *et al*., 2002), *Bacillus subtilis* (Ogura *et al*., 2002), *Campylobacter jejuni* (Takata *et al*., 2005) and *Streptococcus pneumoniae* (Berge *et al*., 2003). In *Haemophilus influenzae dprA* mutants, DNA becomes resistant to extracellular DNA with a similar efficiency as wild-type strains, but the DNA fails to recombine with chromosomal DNA. Interestingly, a *H. pylori dprA* mutant is deficient both in natural transformation with chromosomal DNA as well as with plasmid DNA (Smeets *et al*., 2000), whereas a *H. influenzae dprA* mutant can be naturally transformed with plasmids at nearly wild-type frequency (Karudapuram *et al*., 1995). The latter is reminiscent of a recombination deficiency, because plasmid transformation in *H. influenzae* can occur independent from recombination (Notani *et al*., 1981). In *S. pneumoniae*, it has been shown that in a *dprA* mutant ssDNA that enters the cell is rapidly degraded, a phenotype shared by *recA* mutant strains (Berge *et al*., 2003). Yet although both proteins appear necessary for the protection of incoming DNA during transformation, only RecA seems necessary for homologous recombination between different loci on the chromosome (Berge *et al*., 2003). Furthermore, in contrast to *recA* strains, a *dprA* mutant is not impaired in UV-survival like *recA* mutants of *H. influenzae* (Tomb *et al*., 1989), *S. pneumoniae* (Berge *et al*., 2003) or *H. pylori* (data not shown). Members of the *dprA* gene family, also called *smf*, appear to be highly conserved in bacteria as well as in a single archaeon, *Pyrococcus furiosus*. Only some obligate (intracellular) pathogens such as the *Rickettsiae, Chlamydiae* and *Mycoplasmata*, lack a *dprA* homologue. Despite this conservation, a function for this gene has never been established in non-competent species. A possible explanation is that natural transformation and other forms of DNA transfer might be more widespread among bacteria

than is generally considered, but it is also possible that DprA has other functions beside transformation (Takata *et al*., 2005). To determine whether DprA has a role in DNA repair or recombination we studied the effect of a *dprA* mutation in *Escherichia coli*.

Materials and Methods

E. coli and H. influenzae strains. E. coli strains (Table 1) were grown in Luria-Bertani (LB) medium with thymidine, or in minimal medium (MM) containing per liter: 0.2 g MgSO4.7H2O, 2 g citric acid, 10 g K2HPO4, 3.5 g Na(NH4)HPO4.4H2O, 4 g glucose and 10 mg thiamine. When appropriate, antibiotics were added in the following concentrations (mg/l): kanamycin 50, ampicillin 30 (*E. coli*), 2 (*H. influenzae*), chloramphenicol (cam) 10, tetracycline (tet) 12.5, streptomycin (strep) 25, naladixic acid 8. *H. influenzae* strains (Table 1) were grown in brain heart infusion liquid media supplemented with 2-10ug/ml NAD and 10ug/ml hemin (sBHI) (Barcak *et al*., 1991). sBHI agar medium contained 15 g/l Difco Bacto agar. For growth experiments with *E. coli*, 10ml of medium was inoculated with 10µl of an overnight culture in LB and incubated at 37°C with continuous shaking.

Complementation of H. influenzae. The *E. coli* K12 *smf* gene was obtained by PCR using the primers SMF1e (CGC TCG TCC GGA ATA TGT AA) and SMF2e (TTG ATC CAC ACG CAA CTC AG) and cloned into the pGEM-Teasy vector. Subsequently, a DNA fragment containing the *smf* gene was isolated by digestion with XbaI and EcoRI (the EcoRI site was located on the vector, the XbaI site is present in the *E. coli* genome just in front of the CDS) and ligated into similarly digested pMMB67HE (Furste *et al*., 1986), placing *smf* under control of the tac promoter. The resulting vector was designated pMMB67HE-SMF. This vector was then transferred to *H. influenzae* strains by electroporation and selection for ampicillin resistance.

Construction of smf mutants in E. coli. The method described by Datsenko & Wanner (Datsenko and Wanner, 2000) was used to disrupt the *smf* gene in *E. coli*. Briefly, using primers SMF3 (GCT GCG ATC CAT CCT GCT AAC TCC AGT TCG AGT AGT TGA GTA ACT ACC TCG TGT AGG CTG GAG CTG CT TC) and SMF4 (ATC ACT GAC CAA TCG CAA AGA TTG CTA AGG CTG CTT ATG GCA GGG AGA TAC ATA TGA ATA TCC TCC TTA G) and template pKD3 (Datsenko and Wanner, 2000) a DNA fragment was generated by PCR that contained the cat gene flanked by directly repeated FRT sites and a 50-nt homology extension of the DNA regions flanking the chromosomal *smf* gene. The homology regions were chosen such that the complete reading frame of the *smf* gene was disrupted. After λRED-mediated recombination in strain BW25113 containing pKD46, a

TABLE 1: strains and plasmids used in this study

strain	**Relevant genotype**	**source**
KW20	*H. influenzae* wildtype	(Wilcox and Smith, 1975)
GBH37	*H. influenzae dprA* mutant	(Karudapuram *et al.*, 1995)
Map7	*H. influenzae* wildtype	(Tomb *et al.*, 1989)
GBH37 pMMB67HE	GB37 with empty pMMB67HE	this study
GBH37 pMMB67HE-SMF	GB37 with *smf* on pMMB67HE	this study
KA797	Wild-type	GM1 (Coulondre and Miller, 1977)
KA1808	KA797 *smf*::FRT	this study
PP1573	Wild-type (*thi, del pro-lac,* $strep^R$)	(Groenen *et al.*, 1985)
KA1847	PP1573 *smf::cam*/FRT (cam^R)	this study
CS5542	*cho::cam* (cam^R)	(Moolenaar *et al.*, 2002)
PC4020	Hfr (Tet^R)	NCCB
KA1850	PC4020 *smf::cam* /FRT (cam^R)	this study
CS5017	*uvrB*	(Moolenaar *et al.*, 1994)
KA1843	CS5017 *smf::cam* /FRT (cam^R)	this study
CS5553	*polB*::FRT	N. Goosen, unpublished
KA1844	CS5553 *smf::cam* /FRT (cam^R)	this study
CS5605	*uvrC*::FRT	N. Goosen, unpublished
KA1846	CS5605 *smf::cam* /FRT (cam^R)	this study
CS5621	*dinB*::FRT	(Moolenaar *et al.*, 2005)
KA1845	CS5621 *smf::cam* /FRT (cam^R)	this study
KA1840	CS5628 *uvrC*::FRT, *dinB*::FRT, *polB*::FRT	this study, derived from CS5605
KA1841	KA1840 *smf::cam* /FRT (cam^R)	this study

strain was created in which the mfd gene was replaced by the FRT-flanked cat gene. The construct was verified by PCR with primers SMF5 (TCT TGA TCC ACA CGC AAC TCA GCT TCT G) and SMF6 (TTA GCA ACT TTG CGA AGC CGC TCG TCC) and subsequent restriction of the amplimer with PvuII, which cuts the 1225 basepair (bp) amplimer in three fragments of 90, 525 and 610 bp. The 1355 bp amplimer of the intact *smf* gene was cut in two fragments of 110 bp and 1245 bp (results not shown). The *smf* disruption was transferred to different strains (listed in Table 1) using P1 transduction. Subsequently the cat gene was eliminated from the chromosome using pCP20 expressing the FLP recombinase (Datsenko and Wanner, 2000), resulting in a *smf* deletion with an FRT scar sequence.

Natural transformation of H. influenzae. Natural transformation of *H. influenzae* was performed as described by Barcak *et al*. (Barcak *et al*., 1991). Cells were diluted to an OD650 of 0.0125 in sBHI and grown at 37°C in a shaking water bath at 120 rpm until an OD650 of 0.2-0.3. 17.5 ml of the cell culture was harvested by centrifugation and washed with 5 ml pre-warmed MIV medium, pelleted and resuspended in 17.5 ml fresh prewarmed MIV medium with or without 1 mM IPTG, and again incubated in a shaking water bath at 37°C for 100 minutes at 72 rpm. Subsequently, 1 ml of culture was transferred to a tube with 1 µg of *H. influenzae* strain Map7 DNA and incubated stationary at 37°C for 30 minutes. Dilutions were plated in 45°C molten sBHI agar, and overlayed with either sBHI agar alone or supplemented

with nalidixic acid. Transformation frequencies were determined by dividing cfu/ml on nalidixic acid-containing media by viable cell counts plated on sBHI alone.

Plasmid transformation of E. coli. pUC18 DNA was prepared according to the miniprep spin protocol (Qiagen) and dissolved in milliQ water. Plasmid transformation of *E. coli* was performed as described by Baur *et al*. (Baur *et al*., 1996). In brief, bacteria were grown to an OD600 of 0.4. 1 ml of the culture was centrifuged and resuspended in 100 µl 50µM CaCl2 with 10 ng DNA and incubated on ice for 20 minutes and subsequently at 37°C for 10 minutes. 500 µl LB was added, the bacteria were incubated at 37°C with shaking for 45 minutes, centrifuged and plated on selective media (undiluted and 1:102) and non-selective media (1:106 and 1:108).

Chromosomal transformation of E. coli. chromosomal donor DNA was prepared from 10 ml overnight culture by CTAB (5% hexadecyl trimethyl ammonium bromide) and phenol-chloroform-isoamylalcohol extraction and diluted to 10 ng/µl in 10mM Tris-HCl. The DNA was sonicated for 2 minutes (amplitude 21%). Bacteria were grown to an OD600 of 0.4. 10 ml of culture was mixed with 1 ml 1 M CaCl2 and incubated on ice for 30 minutes. Then the culture was centrifuged, resuspended in 450 µl 100 mM CaCl2 and 50 µl of the sonicated chromosomal DNA was added, incubated on ice for 30 minutes and heat-shocked at 42°C. Then 750 µl of LB was added, the bacteria were incubated at 37°C with shaking for 45 minutes, centrifuged and plated on selective media (undiluted and 1:102) and non-selective media (1:106 and 1:108).

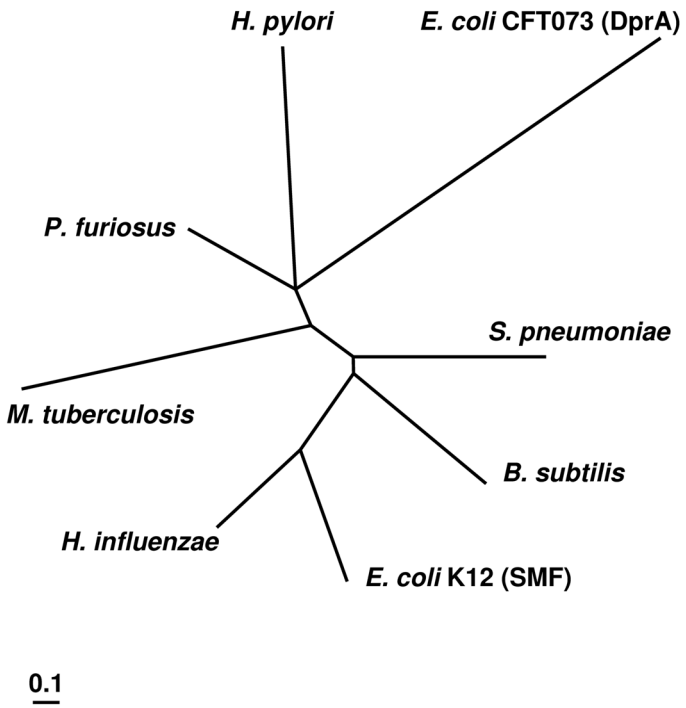

FIG. 1: phylogenetic tree showing the relative distances between translated *dprA* and *smf* CDSs of *H. pylori, H. influenzae, B. subtilis, S. pneumoniae, E. coli smf, E. coli dprA* and *P. furiosus*. The alignment was made with ClustalX 1.83 using the Blosum amino-acid weight matrix. The maximum-likelihood tree was constructed with Tree-Puzzle 5.2 and Treeview 1.6.6. The 50 N-terminal amino-acid residues of *E. coli* CFT073 DprA were excluded from the analysis.

Conjugation of E. coli. Donor and recipient strains were grown to an OD600 of 0.3. 4 ml of the donor strain was mixed with 4 ml of the recipient strain and incubated at 37°C without shaking for one hour. Then the suspension was briefly centrifuged and the bacteria were plated as 1:102, 1:104 and 1:106 dilutions with appropriate selection.

TABLE 2: transformation of *E. coli smf* mutants with *E. coli* CS5542 (cam^R). DNA: donor DNA double stranded (ds) or single stranded (ss). MMC: incubation with Mitomycin C (0,25 mg/l).

Acceptor strain	DNA	MMC	frequency
KA797 wildtype	ds	No	$2*10^{-6}$
KA1808 SMF::FRT	ds	No	$2*10^{-6}$
KA797 wildtype	ds	yes	$5*10^{-6}$
KA1808 SMF::FRT	ds	yes	$5*10^{-6}$
KA797 wildtype	ss	No	$0.7*10^{-6}$
KA1808 SMF::FRT	ss	No	$2*10^{-6}$
KA797 wildtype	ss	yes	$0.4*10^{-8}$
KA1808 SMF::FRT	ss	yes	$1*10^{-8}$

Results and discussion

The E. coli K12 smf gene complements dprA in H. influenzae. *E. coli* contains two *dprA* family members. One of these, called *smf*, is present in all three *E. coli* strains that have been sequenced completely. This gene is highly similar to the *H. influenzae dprA* (44% identity at the amino-acid level). The gene product of the other homolog, called *dprA*, has an additional N-terminal domain of 50 amino-acid residues, which is absent from all other DprA/Smf proteins. Even when this domain is neglected, the amino-acid sequence shares less sequence identity with other DprA/Smf proteins as compared to *E. coli* Smf (figure 1). Moreover, whereas the *E. coli* O157:H7 and *E. coli* CFT073 genomic sequences contain both genes, *E. coli* K12 only contains the *smf* CDS. Therefore, we decided to study the function of *dprA*-like genes in *E. coli* using the *smf* gene in strain K12. A phylogenetic tree of DprA/SMF family members shows no distinction between the DprA/SMF proteins of species with a known DNA-uptake system for natural transformation (*H. influenzae*, *H. pylori*, B. subtilis, S. pneumoniae) and those of species that are not known to be competent for genetic transformation (*E. coli*, *P. furiosus, M. tuberculosis*) (fig. 1). Therefore, it is likely that the role of DprA in natural transformation is not its primary function, similar to RecA and its role in transformation.

To test the hypothesis that DprA/Smf proteins are functionally similar in naturally competent and non-competent species and to establish that the *smf* gene in our *E. coli* K12 strain encodes a functional protein, the *E. coli* K12 *smf* gene was introduced into a *H. influenzae dprA* mutant. The *E. coli* gene was amplified by PCR, cloned in the broad host-range vector pMMB67HE and introduced into wild-type and *dprA* mutant of *H. influenzae*. For transformation experiments with the complemented strain the gene was expressed under

control of the tac promoter. The results are shown in fig 2. As was expected from earlier studies, the transformation frequency of the *H. influenzae dprA* strain was 3 per 109 bacteria, more than 2,000 times lower than the wild-type frequency of 7.2 per 106. After introduction of the *E. coli smf* gene and induction with IPTG the mutant showed natural transformation at a frequency of 2.5 per 107, which is 3.5% of the wild-type frequency and over 80 times more efficient than the *dprA* mutant. The effect of the complementation was weaker in the absence of IPTG, which is probably due to low activity of the tac promoter under this condition because of the presence of the lacIq gene on pMMB67EH. There was no effect of an empty pMMB67HE vector on the transformation efficiency of *H. influenzae*. These data confirm that *E. coli smf* encodes a functional protein which partially restores natural transformation. We conclude that the *dprA*/*smf* genes of naturally competent and non-competent species can be functionally interchangeable.

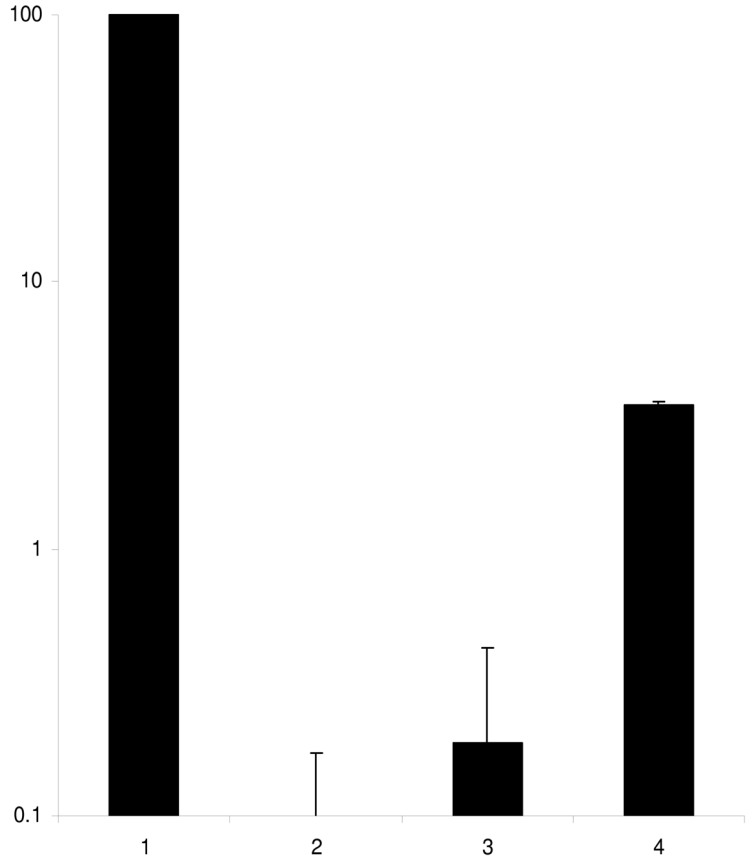

FIG. 2: transformation efficiency of H. influenzae dprA mutants as a percentage of the wild-type (strain KW20) frequency. The bars represent the mean of four experiments performed on 2 days. Bar 1: wild-type KW20. Bar 2: GBH37 pMMB67HE (dprA mutant, empty plasmid) with IPTG. Bar 3: GBH37 pMMB67HE-SMF (dprA mutant, E. coli smf) without IPTG. Bar 4: the same as bar 3, with IPTG.

Transformation of E. coli. Next, we tested whether *E. coli* transformation was affected by the *smf* mutation. *E. coli* is not known to be naturally competent for transformation with chromosomal DNA, but contains an uptake system for the use of DNA as a nutrient (Palchevskiy & Finkel, 2006) and can also be transformed with small plasmids under physiologic circumstances (Baur *et al*., 1996). We did not detect any difference in plasmid transformation frequency between *E. coli* strain KA797 and the *smf* mutant KA1808 (not

shown). Attempts to transform *E. coli* with chromosomal DNA were unsuccessful; DNA of *E. coli* strain CS5542 yielded <1*109 transformants. Therefore, a heat-shock protocol was used for transformation with chromosomal DNA. The transformation frequency was increased if the chromosomal DNA had been sheared by sonication, and was even slightly higher if recombination was stimulated by adding Mitomycin C (MMC) to the growth medium. Heat-shock transformation was performed with double-stranded DNA (dsDNA) as well as with heat denatured single-stranded DNA (ssDNA). In naturally competent species, the DNA is usually single stranded. The transformation frequencies varied widely between individual experiments, but no reproducible differences between wild type cells and the *smf* mutant cells were observed. The results of a representative experiment are given in Table 2. We conclude that *smf* does not play a role in transformation of *E. coli*, despite its ability to restore natural transformation in *H. influenzae*. Although these results might reflect differences between natural transformation and artificial transformation, they are still in contrast to experiments in *C. jejuni*, where a *dprA* mutation abolished the capability of electro-transformation as much as natural transformation (Takata *et al.*, 2005).

Conjugation. A possible role for *smf* in conjugation was investigated by Hfr conjugation experiments, using both wild-type and the *smf* mutant cells as donor as well as recipient strain. Hfr conjugation was chosen because it involves more recombination steps than conjugation of IncP or IncQ plasmids. As can be seen from the results presented in Table 3, the *smf* mutation did not significantly influence conjugation efficiencies, either in the donor or in the recipient. Therefore, Smf does not play a crucial role in plasmid conjugation or plasmid recombination after conjugation.

TABLE 3: conjugation of *E. coli* strains PC4020 and pp1573.

donor	recipient	frequency
PC4020 (wildtype)	pp1573 (wildtype)	$2*10^{-6}$
PC4020 (wildtype)	KA1847 (pp1573 SMF)	$3*10^{-6}$
KA1850 (PC4020 SMF)	pp1573 (wildtype)	$4*10^{-6}$
KA1850 (PC4020 SMF)	KA1847 (pp1573 SMF)	$9*10^{-6}$

Viability and growth characteristics of the smf mutant. DprA/Smf has been reported to play a role in the protection of ssDNA intermediates during transformation (Berge *et al.*, 2003). This function could also be useful in other cellular processes, such as chromosome replication and DNA repair. The growth rate of the *smf* mutant was similar to that of the wild-type (not shown). This suggests that a function in protection of single stranded intermediates during normal chromosomal replication is absent or very limited. Next, we tested the effect of 0.25 mg/l of the alkylating agent MMC on the *smf* mutants in *E. coli* K12. MMC induces point mutations due to mispairing as well as cross-linking of DNA strands at CpG sites, which results in double-strand breaks during replication. This type of DNA damage is lethal unless the double strand break is repaired by homologous

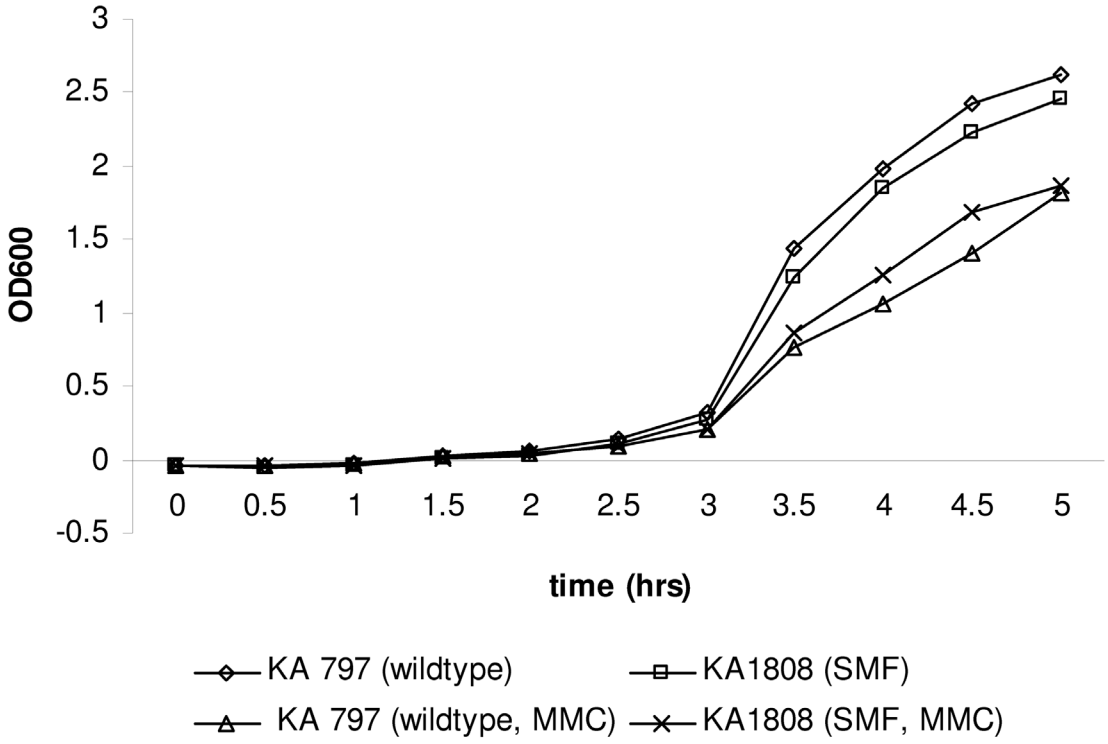

FIG. 3. Growth curve of the smf mutant in the presence and absence of MMC compared to the wild-type.

recombination. As expected, a *recA* strain was unable to replicate when exposed to MMC. However, no differences in growth rate or viability were observed between the wild-type cells and the *smf* mutant (fig. 3). Therefore, we were unable to demonstrate a role of *smf* in the protection of single-stranded intermediates during recombinational DNA repair.

A number of different mechanisms are involved in *E. coli* DNA repair. Because backup-mechanisms (redundancies) may mask a possible effect of *smf* mutation, a combination with other mutations might be necessary to reveal a phenotype. Therefore, we performed a number of experiments that combine *smf*-deletion with mutations in genes involved in DNA repair that might increase the presence of ssDNA. First, we combined a *smf*-deletion with an uvrB or uvrC deletion to eliminate the nucleotide excision mechanism of DNA repair. Nucleotide excision DNA repair does not involve loose ends of ssDNA. Disruption of this system might increase the dependency on DNA repair systems that do involve the formation of ssDNA. Nevertheless, the *smf* deletion did not induce growth deficiencies in these strains either with or without the addition of 0.25 mg/l MMC in the growth medium (not shown). Likewise, UV-irradiation of *smf* mutants (in a wild-type as well as uvrB and uvrC backgrounds) did not reveal an impaired survival. Mutants with deletions in dinB (encoding the mutagenic polymerase IV) and polB, and a combination of dinB, polB and uvrC (see Table 1) were similarly unaffected by an additional *smf* mutation. These results seem to suggest that DprA is not involved in DNA repair or recombination in *E. coli*. Because of the complexity of DNA repair mechanisms in this species, however, this conclusion is premature.

We were not able to establish a role for DprA in horizontal gene transfer via transformation or conjugation in *E. coli*, which raises questions about the supposed basic function of *dprA*/*smf* in these processes. Moreover, a homologue of DprA can also be identified in, for instance, Mycobacterium tuberculosis. This species is thought to have evolved strictly by clonal descent and does not show any signs of recent horizontal gene transfer whatsoever (Brosch *et al*., 2002;Supply *et al*., 2003). The conservation of DprA in many bacterial species strongly suggests that natural transformation is not its main function but that this protein fulfills a role for the fitness of bacteria, but the unraveling of its exact function awaits further experimentation.

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Chapter 5:

comH, a novel gene essential for natural transformation of *Helicobacter pylori*.

Smeets LC, Bijlsma JJ, Boomkens SY, Vandenbroucke-Grauls CM, Kusters JG. J Bacteriol. 2000 Jul;182(14):3948-54.

Abstract

Helicobacter pylori is naturally competent for transformation, but the DNA uptake system of this bacterium is only partially characterized and nothing is known about the regulation of competence in *H. pylori*. To identify other components involved in transformation or competence regulation in this species, we screened a mutant library for competence-deficient mutants. This resulted in the identification of a novel, *Helicobacter* specific competence gene (*comH*) whose function is essential for transformation of *H. pylori* with chromosomal DNA fragments as well as with plasmids. Complementation of *comH* mutants in *trans* completely restored competence. Unlike other transformation genes of *H. pylori, comH* does not belong to a known family of orthologous genes. Moreover, no significant homologs of *comH* were identified in currently available databases of bacterial genome sequences. The *comH* gene codes for a protein with a N-terminal leader sequence and is present in both highly competent and less efficient transforming *H. pylori* strains. A *comH* homolog was found in *Helicobacter acinonychis*, but not in *H. felis* and *H. mustelae*.

Introduction

Helicobacter pylori is a Gram-negative bacterium that colonizes the human stomach and causes chronic gastritis and peptic ulceration. Furthermore, colonization with this organism is associated with the development of gastric neoplasms. More than half of the *H. pylori* strains contain a pathogenicity island, the *cag* region, whose presence has a marked influence on the virulence of the organism.

Gene transfer between *H. pylori* strains is extremely common (25) and can generate novel subtypes during colonization with multiple strains (15;20). The genetic recombination between *H. pylori* strains includes changes in important virulence markers such as the *cag* status (15). Therefore, horizontal gene transfer and uptake of foreign DNA play an important role in virulence and host adaptation of *H. pylori*. Horizontal gene transfer can occur via conjugation, transduction or transformation. Most *H. pylori* strains are naturally competent for transformation with linear DNA (18;28) as well as with plasmids (32). In order to get insight in the characteristics of natural transformation in *H. pylori*, it is necessary to understand the mechanisms involved and their regulation.

When Tomb *et al*. published the first genomic sequence of *H. pylori*, based upon sequence homologies a number of potential competence genes could be recognized. However, no integral DNA-uptake system was identified (26). At present, only for two loci a role in transformation has been described: the comB operon (13) and *dprA* (3;23). To identify other components involved in competence or its regulation, we screened a mutant library for competence-deficient mutants. This resulted in the identification of a novel *H. pylori* competence gene, *comH*. Unlike comB and *dprA*, *comH* does not belong to a known family of orthologous genes.

Materials and methods

Bacterial strains, plasmids and culture media. Bacterial strains and plasmids are listed in table 1. *H. pylori, H. mustelae* (kindly provided by T. Ó Cróinín, Our Lady's Hospital for Sick Children, Crumlin, Ireland) and *H. acinonychis* (kindly provided by A. Bart, Academic Medical center, Amsterdam, the Netherlands) were grown under microaerobic conditions on Dent plates (9) (Dent supplement: Oxoid, GB) supplemented with 40 mg of 2,3,5-triphenyltetrazolium chloride (Sigma Chemical Co., St. Louis, Mo., USA) per liter. When appropriate, antibiotics were added in the following concentrations: kanamycin, 20 mg/l

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or characteristics	Reference or source
H. pylori 26695	wild type	(26)
H. pylori SPM326	wild type	(17)
H. pylori SS1	wild type	(16)
H. pylori HPK1	wild type	(28)
H. pylori NCTC 11637	wild type	NCTC[1]
H. pylori J99	wild type	(1)
H. pylori ATCC 43504	wild type	ATCC[2]
H. pylori BR9802	wild type	(31)
H. pylori 1061	wild type	(11)
1061, SACHA-1	1061, HP1527::aphA-3	this study
1061, SACHA-2	1061, HP1527::aphA-3	this study
26695, SACHA-1	26695, HP1527::aphA-3	this study
H. felis CS1	wild type	CCUG[3] 28539
H. felis DS4	wild type	CCUG 28540
H. mustelae NCTC 12198	wild type	NCTC
H. mustelae NCTC 12032	wild type	NCTC
H. mustelae F4	wild type, ferric isolate	T. Ó Cróinín[4]
H. mustelae F8	wild type, ferric isolate	T. Ó Cróinín
H. mustelae F9	wild type, ferric isolate	T. Ó Cróinín
H. acinonychis India	wild type	(6)
H. acinonychis Sheeba	wild type	(6)
H. acinonychis ATCC 12686	wild type	(6)
HpC-1527	26695, *rdxA*::HP1527. Mtz^R	This study
HpC-SACHA	26695, HP1527::aphA-3, *rdxA*::HP1527. Mtz^R, Km^R	This study
pBC SK^-	Phagemid, derivative of pUC19	Stratagene
pRdxA	pBC-SK^-, multiple cloning site flanked by the 5'and 3' part of *rdxA*	This study
pRDXA-1527	pRdxA, intact gene HP1527	This study
pSACHA-1	pGEM-T easy, HP1527::aphA-3	This study
pSACHA-2	pGEM-T easy, HP1527::aphA-3	This study
E. coli ER1793	Host strain	(14)
E. coli DH5α	Host strain	Clontech
pGEM-T easy	PCR cloning vector. ColE1, Amp^R	Promega
pHel2	*H. pylori* - *E. coli* shuttle plasmid. ColE1, Cam^R	(12)
pJMK30	pUC19. Amp^R, Km^R	(30)
pBCα3	*H. pylori* suicide vector. ColE1, Cam^R, Km^R	(4)

[1] NCTC: National Collection of Type Cultures, Colindale, UK

[2] ATCC: American Type Culture Collection, Manassas, Va., USA

[3] CCUG: Culture Collection Univerity of Göteborg, Göteborg, Sweden

[4] T. Ó Cróinín, Our Lady's Hospital for Sick Children, Crumlin, Ireland

(Sigma); chloramphenicol, 15 mg/l (Serva, Heidelberg, Germany); and clarithromycin, 2 mg/l (Abbott Laboratories Ltd., Queensborough, GB). *H. felis* strains were grown as described by Cattoli *et al*. (7). *Escherichia coli* ER1793 (14) and DH5α (Clontech, Palo Alto, Ca., USA) were cultured in Luria Bertani broth, with 30 mg/l kanamycin or 30 mg/l chloramphenicol if appropriate. Plasmid pHel2 is an *E. coli – H. pylori* shuttle vector that carries the Cat_{GC} chloramphenicol resistance gene (12). The pBCα3 suicide vector was derived from the pBC SK+/- plasmid (Stratagene, La Jolla, Ca, USA) by ligation of the *aphA-3* kanamycin resistance cassette (27) into its unique *Sma*I site (4).

TABLE 2. Oligonucleotide primers used in this study

Primer designation	Annealing site	Primer sequence (5'-3')
AphA3-R	gene aphA-3 base 1381-1401	CTGGATGAATTGTTTTAGTAC
Kana-L	gene aphA-3 base 36-16	TTACCTATCACCTCAAATGG
HP1526rev62	ORF HP1526 base 62-41	TCCATAAAGCCCTTAGTCAT
HP1527for43	ORF HP1527 base 43-62	AACCCTCTTCAAGCCCTTGT
HP1527rev1156	ORF HP1527 base 1156-1137	CCAATTCGCTGGTTTCATAA
HP1529for1110	ORF HP1529 base 1110-1129	CAAAGTCTCTTCGCGCCAAA
HP1529for1323	ORF HP1529 base 1323-1342	CCGCTTGAACGAATTGAACG
MetroF	ORF HP0955 base 751-770	AATTTGAGCATGGGGCAGA
pUC/M13 forward	pGEM-T	GTTTTCCCAGTCACGAC
pUC/M13 reverse	pGEM-T	CAGGAAACAGCTATGAC
rdxAISacI	ORF HP0955 base 774-789	TTTgagctcATTTATGGTAG [a]
rdxAIXbaI	ORF HP0954 base 322-342	CACtctagaCTTATAAGACTCC
rdxAIIXhoI	ORF HP0954 base 384-366	TTGctcgagTGCTTGGCG
rdxAIIKpnI	ORF HP0954 base 613-635	ATCggtaccAAGTAATCGCATC

[a] Lower case letters represent restriction sites that were introduced into these primers.

DNA manipulation. Southern blotting and recombinant DNA techniques were performed according to standard protocols (21) unless stated otherwise. Plasmids were isolated with the QIAprep spin miniprep kit (QIAgen GmbH, Hilden, Germany). Restriction enzymes used in this study were obtained from New England Biolabs Inc. (NEB, Beverly, Massachusetts, USA).

Transformation. Natural transformation of *H. pylori* was performed essentially as described by Wang *et al.* (32). In brief, 24 hours after inoculation bacteria were harvested from their plate and transferred as thick patches on a fresh plate, after 5 hours approximately 1

µg of DNA was added to the patches. After 20 hours of incubation, the bacteria were suspended in 120 µl of PBS and 100 µl of appropriate dilutions were spread on selective plates. To calculate a transformation frequency, appropriate dilutions (10^{-6} and 10^{-8}) were plated on non-selective plates. After incubation for 5 days, the colonies were counted.

Electrocompetent *H. pylori* cells were prepared as described for *Campylobacter jejuni* (29). Electroporation was performed on a ECM-600 Electroporation system (BTX, San Diego, Ca., USA) with 50 µl of competent cells and 1 µg salt-free DNA at 12.5 kV cm^{-1} and 50 µF. The bacteria were suspended in 1 ml of Brucella broth containing 2% newborn calf serum and 0.4% Dent supplement immediately after electroporation, plated on non-selective plates within 15 minutes and allowed to recover during 7 hours of microaerobic incubation. Thereafter they were transferred to selective plates.

Construction and screening of the library. The construction of the *H. pylori* mutant library has been described before (4). Individual mutants of the library were inoculated as patches on kanamycin-agar. After 24 hours of growth, the patches were covered with 10 µl of a 25 ng/µl chromosomal DNA solution that confers clarithromycin resistance due to a A-2142 to G mutation in the 23SrDNA (8). After another 24 hours of growth, the patches were transferred to plates containing clarithromycin.

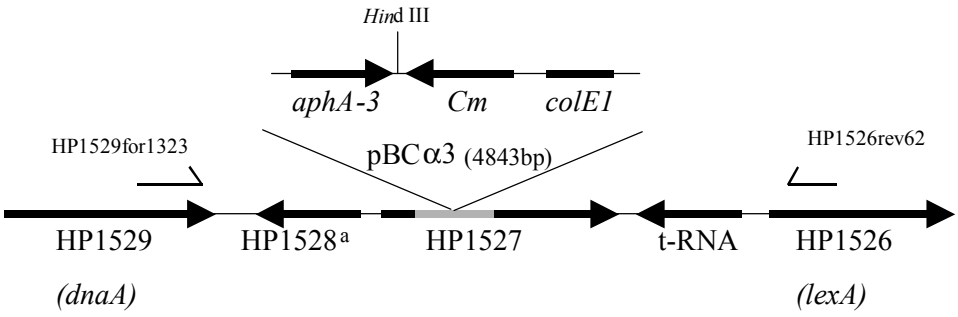

FIG. 1. Schematic representation of the genomic region of HP1527 with the location of the pBCα3 insertion. Depicted are relevant regions of pBCα3 and the *Hin*d III site used for plasmid rescue, the duplicated chromosomal region (gray) and the primers used for complementation of HP1527 (Table 2). The figure is not drawn to scale. *AphA-3*: kanamycin resistance cassette. *Cm*: chloramphenicol resistance cassette. *colE1*: origin of replication.

[1)] HP1528 is present in strain 26695 only

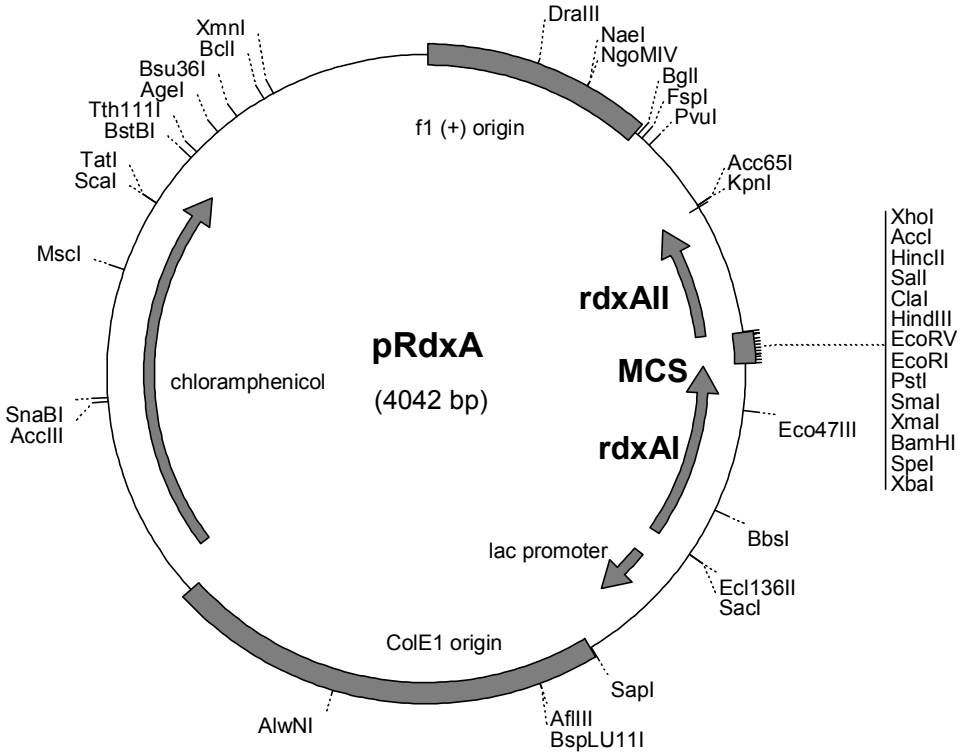

FIG. 2. Map of vector pRdxA. The multiple cloning site (MCS) allows for cloning between two fragments of the *rdxA* gene (rdxAI and rdxAII). Relevant restriction sites are indicated.

Plasmid rescue, sequencing and sequence analysis. For plasmid rescue chromosomal DNA of the mutants was isolated and restricted with *Hin*d III (a unique *Hin*d III restriction site is present on pBCα3 between the *aphA-3* kanamycin and chloramphenicol resistance cassettes, see fig.1), self-ligated into circularized *Hin*d III fragments and transformed into *E. coli* with selection on chloramphenicol. For the determination of the other point of insertion the circularized *Hin*d III fragments were used as template in an inverse PCR reaction with primers that face outward on the *aphA-3* cassette: AphA3-R and Kana-L (Table 2). PCR-products were cloned in the pGEM-T Easy vector (Promega, Madison, WI, USA).

Sequence reactions were then performed on the rescued plasmids and on the cloned inverse-PCR amplimers with the Thermo-Sequenase pre-mixed cycle sequence kit (Amersham Pharmacia, Uppsala, Sweden) with standard M13 primers (Texas Red labeled) on an Amersham Vistra 725 sequencer. Data were analyzed with Lasergene software (DNAstar

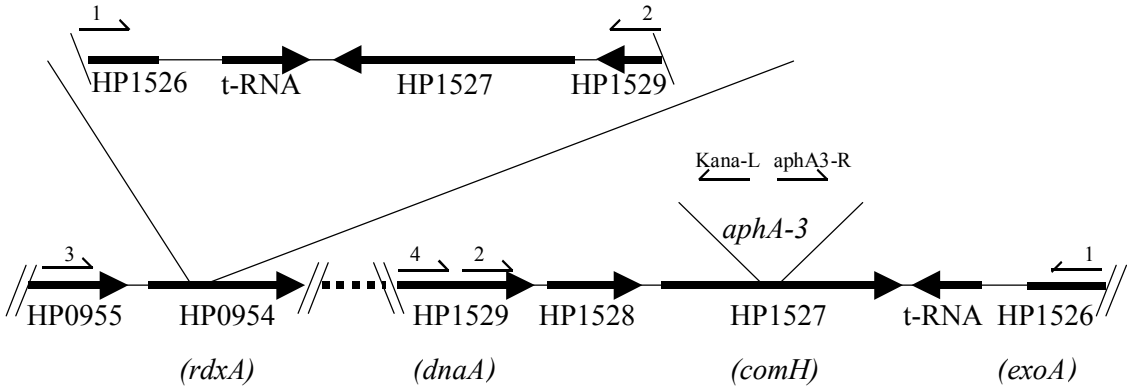

FIG. 3A. Schematic representation of complementation mutant HpC-SACHA: the *rdxA* region with HP1527 insert (left side) and the HP1527 region with *aphA-3* insert (right side). Indicated are the primers used to construct HpC-1527 and to confirm the site of *aphA-3* insertion. Primers 1 (HP1526rev62) and 2 (HP1529for1323) were used to amplify HP1527 from strain 1061 (that lacks ORF HP1528, see text). This HP1527 copy was inserted into the *rdxA* gene of strain 26695. The presence of an uninterrupted HP1527 gene in the *rdxA* gene was confirmed by a PCR with primers 3 (MetroF) and 2. Primers 4 (HP1529for1110) and Kana-L confirmed the *aphA*-3 insertion in the original HP1527 gene, while primer 3 did not yield a product with aphA3-R.

inc. Madison, WI, USA). Sequence analysis was performed with the BLAST2.0 algorithm (2) (National Center for Biotechnology Information, Los Alamos, N.Mex., USA).

Construction of site-directed mutants in ORF HP1527. A fragment of the open reading frame (ORF) HP1527 was amplified from *H. pylori* strain 1061 with primers HP1527for43 and HP1527rev1156 (Table 2) and cloned into pGEM-T easy. This HP1527 fragment contains a *Hin*d III site at base 753 of the ORF, that was used for restriction and subsequent ligation with the *aphA-3* containing *Hin*d III-fragment of pJMK30 (30) and cloned in *E. coli* DH5α to obtain pSACHA-1 and pSACHA-2. The orientation of *aphA-3* in pSACHA-1 (same direction as the HP1527 ORF) and pSACHA-2 (opposite direction) was determined by PCR with combinations of primers aphA3-R or

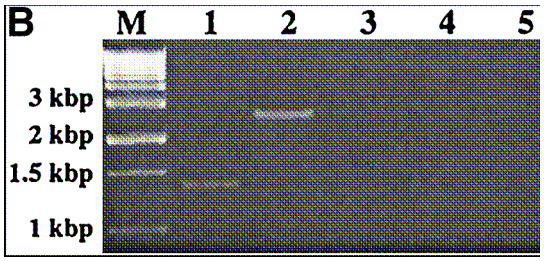

FIG. 3B. PCR reaction confirming the HpC-SACHA genotype in Fig. 3a. Lane 1: primers 4 and Kana-L confirmed the *aphA*-3 insertion in the original HP1527 gene (band 1359 bp). Lane 2: the presence of an uninterrupted HP1527 gene in the *rdxA* gene was confirmed by a PCR with primers 2 and 3 (2410 bp). Lane 3: primer 3 did not yield a product with aphA3-R, confirming the absence of the aphA-3 insertion in the complementing gene copy. Lane 4: control for lane 1 with HpC-1527 template. Lane 5: control for lane 2, with parental strain as template.

Kana-L (forward) and HP1527for43 or HP1527rev1156 (reverse) (Table 2) and sequencing of the amplimers. pSACHA-1 and pSACHA-2 were used to create HP1527 mutants in strain 1061 and 26695 by natural transformation (Table 1).

Construction of the rdxA-vector. The 5'-part of the *rdxA* gene was PCR amplified with the primers rdxAIXbaI and rdxAISacI (Table 2) and the resulting amplimers were purified. This 5'-fragment of *rdxA* was cloned into the phagemid pBC-SK using the *Xba*I and *Sac*I restriction sites that were introduced by PCR, which resulted in the vector pBC-rdxAI. Subsequently the 3'-part of *rdxA* was PCR amplified with the primers rdxAIIXhoI and rdxAIIKpnI (Table 2) and the resulting amplimers were purified. The introduction of this 3'-fragment of *rdxA* into pBC-rdxAI, with the aid of the *Xho*I and *Kpn*I restriction sites that were introduced by PCR, gave rise to the plasmid pRdxA (Fig. 2).

Complementation analysis with the rdxA-vector system. The complete gene HP1527 of strain 1061 was PCR-amplified with primers on the flanking genes: HP1526rev62 and HP1529for1323 (Table 2, Fig. 1). This amplimer was cloned in pGEM-T Easy, and ligated into the *Eco*RI site of the *rdxA*-vector to obtain pRDXA-1527. After cloning into *E. coli* DH5α, pRDXA-1527 was transformed into *H. pylori* strain 26695, this gave rise to the Metronidazole resistant (Mtz^R) mutant HpC-1527. HpC-1527 was transformed with pSACHA-1. The resulting kanamycin resistant (Mtz^R Km^R) colonies were tested for the location of the *aphA-3* cassette with a set of PCR reactions: Apha-L or Apha-3R was used as a forward-primer and reverse-primers were chosen on the *rdxA*-ORF (MetroF) and the HP1529 ORF (HP1529for1323) (Table 2, Fig. 3).

Results

*Screening of the library***.** Approximately 1,250 mutants from a random *H. pylori* 1061 library were screened for transformation deficiency. Each mutant was inoculated as a small patch and, after 24 hours, overlaid with chromosomal DNA that confers clarithromycin resistance. After another 24 hours, the patches were transferred to selective plates. In this crude but easy-to-perform screening method 1,200 mutants formed one or more Cla^R colonies and thus proved to be competent. The remaining fifty mutants were subjected to natural transformation by the method of Wang *et al.*. In this test, three of the fifty were completely transformation deficient and were selected for further examination.

Plasmid rescue, sequencing and sequence analysis. The library that was used for this screening was created by chromosomal insertion of pBCα3 suicide plasmids which contain a random fragment of *H. pylori*. This random fragment of DNA recombines into the *H. pylori*

TABLE 3. Transformation frequencies[a] of wild-type strain 1061, 26695 and HP1527 mutants, with chromosomal DNA and plasmid pHEL2.

Strain, mutant	Transformation frequency[a]		
	Chromosomal DNA, Cla^R		plasmid HEL2, Cam^R
	Natural transformation	Electroporation	
1061, parent	$3 * 10^{-6}$ (100%)	+[b]	$1 * 10^{-6}$ (100%)
1061, SACHA-1	$< 1 * 10^{-9}$ (<0.1%)	+	$< 1 * 10^{-9}$ (<0.1%)
1061, SACHA-2	$< 1 * 10^{-9}$ (<0.1%)	+	$< 1 * 10^{-9}$ (<0.1%)
26695, parent	$1 * 10^{-6}$ (100%)	+	n.d.[c]
26695, SACHA-1	$< 1 * 10^{-9}$ (<0.1%)	+	n.d.

[a] Determined as number of resistant colonies per µg of DNA per recipient CFU, data represent the means of two experiments. Between brackets relative to parental strain.

[b] +: succesfull electroporation

[c] Not determined.

chromosome by a single homologous cross-over event, which leads to insertion of the complete vector and to a duplication of the DNA fragment of the *H. pylori* chromosome. Thus, each mutant contains one copy of this fragment on each side of the integrated vector. Because of this duplication, the backbone of the pBCα3 vector that interrupts the chromosome has a different insertion point on each side. To determine the first point of insertion, plasmid rescue was performed by restriction with *Hin*d III and religation of the chromosomal DNA, which restores a pBCα3-based $Chlor^R$ plasmid that contains one flanking sequence of the *H. pylori* chromosome (Fig. 1). No suitable restriction endonuclease site was available to obtain a rescue plasmid that contains the other flanking sequence. Therefore, the circularized *Hin*d III fragments were used as a template in a reverse PCR reaction with primers that face outward on the *aphA-3* resistance cassette. Thus, the chromosomal DNA flanking the *aphA-3* cassette was amplified.

Both flanking sequences revealed the same site of insertion in the three mutants with a chromosomal duplication of 280 bp. Apparently all three mutants were derived from a single pBCα3 vector, either as independent transformants of the same pBCα3 vector or as offspring from a single mutant that divided before storage. The duplicated region flanking both insertion points was aligned with the complete *H. pylori* genomes of strain 26695 (The Institute for Genomic Research, Rockville, Maryland, USA, http://www.tigr.org) and strain J99 (AstraZeneca R&D, Boston, USA, http://scriabin.astrazeneca-boston.com/hpylori) and was identified as base 550-830 of the ORF designated HP1527 in strain 26695 (JHP1416 in strain J99).

Construction of site-directed mutants in ORF HP1527 and transformation. To prove that the transformation deficiency of the random mutants was not caused by an unrelated event elsewhere in the genome, site-directed mutants were constructed in strain 1061 by insertion of an *aphA-3* cassette in ORF HP1527. First, a fragment of ORF HP1527 of strain 1061 was PCR-amplified and cloned in the pGEM-T Easy vector. Sequence analysis revealed a *Hin*d III restriction site in this DNA fragment. This site was used to insert the *aphA-3* cassette, that codes for kanamycin resistance, and the resulting constructs were named pSACHA-1 and pSACHA-2. PCR reactions with combinations of primers aphA3-R or Kana-L (forward) and HP1527for43 or HP1527rev1156 (reverse) and sequencing of the amplimers showed that pSACHA-1 has the *aphA-3* gene inserted in the same direction as the HP1527 reading frame and pSACHA-2 in the opposite direction. pSACHA-1 and pSACHA-2 were used to create mutants in strain 1061. In addition, a SACHA-1 mutant was also made in strain 26695 to confirm that the phenotype caused by disruption of HP1527 is similar in an unrelated strain. Disruption of ORF HP1527 in each mutant was confirmed by Southern blot (results not shown).

The competence of these mutants was compared to that of their parental strains (Table 3). Both parental strains transformed at a frequency of at least 1 x 10^{-6} with chromosomal DNA conferring clarithromycin-resistance. In contrast, no transformants were observed in the HP1527 mutants, neither with the 1061 mutants nor with the 26695 mutant. As our transformation system detects a transformation frequency of approximately 1 x 10^{-9}, the efficiency of transformation of the mutants is at least 3 log lower than the parental strains. Electroporation of the mutants showed a transformation efficiency comparable to the parental strain. The strain 1061 mutants were also tested for their natural transformation competence with a *H. pylori* plasmid, pHEL2. Again, no transformation was observed (Table 3).

Construction of the rdxA-vector. For the complementation of HP1527 we developed a replacement vector that would allow for the ectopic integration of DNA into the chromosome of *H. pylori*. As a target for replacement we used the gene *rdxA*. Disruption of *rdxA* causes metronidazole resistance in *H. pylori*. Thus, insertion of any DNA fragment into *rdxA* will give rise to metronidazole resistant colonies and the DNA serves as its own resistance marker to select for the successful integration into *rdxA*. For the construction of the *rdxA*-vector, two fragments of the *rdxA* gene were amplified by PCR. With the aid of the restriction sites that were introduced during PCR, the 5'-fragment of the gene was cloned into the first two restriction sites of the multiple cloning site of the phagemid pBC SK- and the 3'-fragment was cloned into the last two restriction sites. This resulted in pRdxA (Fig. 2), a plasmid

containing the 5'- and 3'-part of the *rdxA* gene flanking the remainder of the multiple cloning site, that allows for the introduction of a DNA fragment. Sequencing of pRdxA with the M13 forward and M13 reverse primers, located just outside the multiple cloning site, confirmed the correctness of the inserts. Transformation of pRdxA into *H. pylori* yielded metronidazole resistant colonies, indicating that introduction of the multiple cloning site of pRdxA disrupted the *rdxA* gene (data not shown).

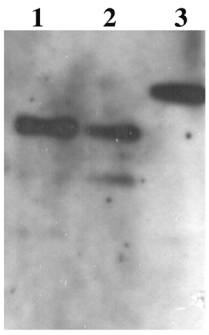

FIG. 4. *H. acinonychis* strains probed with an internal *comH* fragment. Lane 1: strain Sheeba, lane 2: strain India, lane 3: reference strain ATCC12686.

Complementation analysis with the rdxA-vector system. Complementation of ORF HP1527 was performed to confirm that the competence-deficient phenotype of the mutants was caused by disruption of ORF HP1527 and not by a polar effect on surrounding genes. Gene HP1527 was amplified by PCR with primers located on the flanking genes. Strain 26695 contains a small ORF (HP1528) that overlaps the putative promotor region of HP1527. In order to obtain gene HP1527 only and to avoid problems due to this overlap in strain 26695, gene HP1527 was amplified from strain 1061 which lacks ORF HP1528. The amplimer was cloned in the *rdxA*-vector to yield pRdxA-1527 (Fig. 3a). Because disruption of ORF HP1527 eliminates competence, we performed the complementation of HP1527 as follows: first, pRdxA-1527 was transformed to the wild-type *H. pylori* strain 26695. A Mtz^R mutant of 26695 with a second intact HP1527 inserted into the *rdxA* gene, directed opposite to the *rdxA* reading frame, was identified by PCR and called HpC-1527. Next, HpC-1527 was transformed with pSACHA-1, which yielded Mtz^R Km^R transformants with an interruption of either the original or the additional HP1527 ORF. The location of the *aphA-3* insertion was identified with a set of PCR-reactions that demonstrate the presence or absence of the *aphA-3* cassette, both at the original location and in the *rdxA*-gene, as is shown in Fig. 3a and 3b. A mutant with the *aphA-3*-insertion in the original HP1527 was called HpC-SACHA. We then tested the wild-type 26695 and its derivatives HpC-1527, which contains two intact HP1527 genes, and the complemented genotype HpC-SACHA for their capability to transform to clarithromycin resistance. The transformation frequency of HpC-SACHA was identical to the frequency of the parental strain (Table 4). The duplication of gene HP1527 in mutant HpC-1527 had no marked effect on the transformation frequency.

TABLE 4. Transformation frequency[a] of strain 26695 complementation mutants

Mutant	Transformation frequency, between brackets relative to the parental strain
26695, parent	$1 * 10^{-6}$ (100%)
26695 SACHA-1 (HP1527::*aphA-3*)	$< 1 * 10^{-9}$ (<0.1%)
HpC-1527 (HP1527, *rdxA*::HP1527)	$1 * 10^{-6}$ (100%)
HpC-SACHA (HP1527::*aphA-3*, *rdxA*::HP1527)	$1 * 10^{-6}$ (100%)

[a] Determined as number of resistant colonies per µg of DNA per recipient CFU, data represent the means of two experiments.

Distribution of HP1527 in the genus Helicobacter. The nine wild-type *H. pylori* strains from table 1 were tested for the presence of *comH* on a Southern blot probed with a *comH*-fragment (base 62-1156) of strain 1061, and *comH* was demonstrated in all of them (data not shown). These nine strains comprised both highly competent strains and strains with relatively low competence such as SS1. The same *comH* fragment also hybridized to three strains of *Helicobacter acinonychis* (Table 1, fig. 4), a species that is closely related to *H. pylori* and also naturally transformable (results not shown). However, Southern blotting experiments did not demonstrate sequences homologous to *comH* in two other *Helicobacter* species; *Helicobacter felis* (two strains, table 1) and *Helicobacter mustelae* (five strains, table 1).

Discussion

To identify elements of the transformation system in *H. pylori*, we screened a random insertion library for loss of competence. We identified a mutant in ORF HP1527 that was incapable of natural transformation. Site-directed mutants in this ORF showed the same phenotype. Complementation of HP1527 in *trans* completely restored competence, which indicates that the mutation itself rather than a polar effect on surrounding genes causes the transformation deficiency. These results demonstrate that HP1527, an ORF with a heretofore unknown function, is essential for natural transformation of *H. pylori*. Although the gene might have additional functions, based upon the data presented in this paper a gene name in accordance with current nomenclature for competence genes would be appropriate for HP1527. Because the gene has no orthologs (see below), we decided to name HP1527 *comH*, which is, to our knowledge, the first available *com* letter in the alphabet. The *comH* gene is present in all tested *H. pylori* strains, not only in highly competent strains but also in less efficient transformers, and interruption of this gene completely obliterated the natural

transformation competence for both chromosomal DNA as well as for plasmids. *comH*-mutants appear to have a normal growth rate and survival, which suggests that *comH* has no additional household functions.

The organization of the chromosomal region around *comH* differs between strains 26695, J99 and 1061. In all three strains, the *H. pylori exoA* homolog (HP1526) is located downstream of *comH*, separated from *comH* by a t-RNA gene that lies in the opposite direction. In the 26695 sequence, upstream of *comH* are the putative ORF HP1528 and the *dnaA* homolog (HP1529). The small putative ORF HP1528 is absent in 1061 and in the J99 sequence, which indicates that it is not required for competence in *H. pylori*. Because of the large and variable intergenic region between *comH* and the *dnaA* gene, as well as the putative function of *dnaA* in chromosomal replication, co-transcription of *comH* in an operon with this gene is unlikely. Comparison of the ORF *comH* (strain 26695) with the corresponding ORF of strain J99, JHP1416, revealed an amino-acid identity of 93% (95% similarity), which is in line with the variation between other genes in *H. pylori*. Sequence analysis with the SignalP program (19) showed that *comH* has a presumed transmembrane domain corresponding to a N-terminal signal peptide for secretion, with a cleavage site between amino-acid residues 19 and 20. The putative exported mature protein consists of 460 amino acid residues (52.4 kDa) and has an isoelectric point of 6.35. We did not find sequences that may function as DNA-binding sites in *comH*.

In Southern blotting experiments with a *H. pylori comH* probe, a *comH* homolog was detected in all *H. pylori* and *H. acinonychis* strains but not in *H. mustelae* and *H. felis*. Likewise, the latter two species did not hybridize with the *H. pylori* comB operon in earlier experiments by Hofreuter *et al.* (13). Because *H. mustelae* is also naturally transformable (data not shown), these results suggest that transformation genes are not conserved among all naturally transformable *Helicobacter* spp. Database sequence similarity searches did not reveal a significant homology of ORF *comH* to any genomic sequence available in GenBank, including naturally transformable species like *Bacillus subtilis, Haemophilus influenzae* and *Campylobacter jejuni*. This indicates that part of the *H. pylori* transformation system is evolutionary distinct from the systems known from other species. The previously identified comB and *dprA* genes, however, have orthologs in other competent bacterial species and even in conjugational plasmids (26).

H. pylori contains many ORFs without an obvious orthologue and the screening of a random library is therefore a powerful method for the identification of gene function.

Although a previous screening of this library revealed 8 unique mutants (4), the present identification of three identical clones indicate that the 1,250 insertion mutants are not all independent. In addition to this, not all pBCα3 insertions a will inactivate a gene and only one restriction enzyme was used to create the random fragments for mutagenesis. The present set of mutants is therefore not a comprehensive library of the 1,500 *H. pylori* genes. Indeed, none of the known transformation genes (comB operon, *recA* and *dprA*) was identified in our screening. It is therefore possible that other unrevealed competence genes are present.

In this paper we also describe a new complementation strategy for *H. pylori*, based upon the *rdxA*-vector. This complementation system has obvious advantages over plasmid-based complementation: it produces a stable, single-copy insertion. Furthermore, the *rdxA* vector allows for introduction of DNA into *H. pylori* with an absolute minimum of changes in the genome: it avoids the unknown effects of using additional resistance markers that are unnatural to *H. pylori* and does not introduce remnants of the vector other than a short polylinker sequence. Many clinical isolates of *H. pylori* are metronidazole resistant, which indicates that disruption of *rdxA* does not have a significant effect on the viability of *H. pylori*. A practical advantage of the lack of an additional resistance marker is the reduced length of the DNA fragment that has to be internalized, which enhances the transformation frequency in less competent strains.

The lack of orthologs makes it difficult to speculate on the role of *comH* in the process of transformation. In general, natural transformation can be divided into the following steps: development of a competent state, DNA-binding, DNA uptake and genomic integration (24). The putative N-terminal secretion signal of *comH* suggests that the protein is either anchored in the cytoplasmic membrane or exported to the periplasm and points in the direction of a role in the DNA-binding or DNA-uptake process, although a function in the development of a competent state can not be excluded. The results of electroporation experiments, that demonstrate a normal recombination in *comH* mutants, imply that *comH* is not involved in the recombination that follows uptake of chromosomal fragments. This is in accordance with the finding that *comH* mutants are incapable of plasmid uptake, since RecA deficient *H. pylori* mutants are still capable of transformation with self replicating plasmids but not with chromosomal markers (22).

It has become clear from both published genomic sequences that *H. pylori* contains relatively few operonic loci. Whereas the *H. pylori comB* competence genes appear to form a small operon, *dprA* and *comH* do not. Organization of competence genes in larger loci and

operon structures has been described in other naturally transformable bacteria. In *B. subtilis*, the expression of natural transformation competence is a highly regulated process: competence genes are controlled by a complex signal transduction network that senses environmental changes and competence is expressed only under specific circumstances (10). In contrast, *H. pylori* can be transformed under standard culture conditions. The lack of operonic organization of competence genes in *H. pylori* could therefore well reflect a relatively loose regulation of competence, like in *Neisseria* spp. (5). The evidence for extensive horizontal gene transfer between *H. pylori* strains as well as the conserved nature of *comH* and other transformation genes stresses the importance of natural transformation for this organism.

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Chapter 6:

Presence of restriction-modification systems and palindrome avoidance in *Helicobacter pylori*: a comparison of two genomic sequences

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Abstract

In many bacterial as well as phage genomes, palindromic sequences are significantly less abundant than would be expected by chance, a phenomenon known as "palindrome avoidance". According to the restriction hypothesis, palindrome avoidance is caused by restriction-modification systems (RMSs) that use palindromes as recognition sequences. To test this hypothesis, we compared the presence of recognition sequences in the two published *H. pylori* genomic sequences. In contrast to the restriction hypothesis, palindrome avoidance in *H. pylori* is not stronger when the corresponding RMS is present than when it is absent. Our data suggest that the restriction hypothesis can not explain palindrome avoidance in *H. pylori*.

Introduction

Type II Restriction modification systems (RMSs) consist of a combination of a site-specific endodeoxyribonuclease or restriction enzyme (RE), and a DNA methyltransferase (DMT) that offers protective methylation to the recognition site of the RE. The recognition site is often a palindromic sequence of 4-8 nucleotides. Initially, it was proposed that RMSs could serve as a bacterial defense mechanism against bacteriophage attack (Arber, 1965; Bickle & Kruger, 1993). However, the existence of infrequently cutting enzymes, with recognition sequences of 8 bp, is insufficiently explained if their usual target DNA is a small genome such as that of a phage. They can only affect a genome large enough to contain at least a few 8-bp recognition sites, such as that of a bacterium (Naito *et al.*, 1995). Furthermore, RMSs attack double-stranded (ds) DNA whereas many phages are single-stranded (ss). It has been shown that RMSs might also serve as a so called 'addiction mechanism' by unstable genetic elements like plasmids, or an RMS can simply exist as selfish DNA. If a RMS is lost from the genome, the RE will remain active long enough to kill the host organism by restriction of its DNA because the RE is more stable then the DMT (Kusano *et al.*, 1995; Handa *et al.*, 2000).

In many bacterial as well as phage genomes, some (but not all) palindromic sequences of 4 to 6 bp are significantly less abundant than would be expected by chance (Sharp, 1986; Gelfand & Koonin, 1997; Rocha *et al.*, 2001), a phenomenon that is also known as "palindrome avoidance". Palindrome avoidance suggests that RMSs pose a selective pressure against their recognition site in host strains. There are, however, some observations that contradict this theory. For instance, in some bacterial species (e.g. *Chlamydia* spp), palindrome avoidance also occurs in the absence of any known RMS. Palindromes can be avoided while corresponding RE's are not described in the same species (Gelfand & Koonin, 1997). On the other hand, some RMSs do not seem to induce any palindrome avoidance whatsoever.

H. pylori is a species for which two complete genome sequences of different strains are available. In addition, the specificities of the RMSs of both strains are known, as well as those of other *H. pylori* RMSs (Lin *et al.*, 2001; Vitkute *et al.*, 2001). In this study, we related palindrome avoidance in the two published *H. pylori* genomes to the presence or absence of the corresponding RMS, to test the hypothesis that a palindrome is more strongly avoided in a strain when the corresponding RMS is present than in its absence.

Methods

We searched the complete genomic sequences of *H. pylori* strain 26695, [GenBank:AE000511] (Tomb *et al.*, 1997) and J99 [GenBankAE001439] (Alm *et al.*, 1999) for the presence of all possible tetrameric, pentameric and hexameric oligonucleotide sequences with the SWAAP 1.0.0 program (Pride *et al.*, 2003) available at www.bacteriamuseum.org/SWAAP/SwaapPage.htm. The observed number of occurrences of such sequences was compared to the expected number with the Markov-chain method implemented in the SWAAP program (Pride *et al.*, 2003) and expressed as the observed/expected (obs/exp) ratio.

Results

The RMSs in H. pylori strain 26695 and strain J99. All twenty-four *H. pylori* RMSs presently known are summarized in Table 1. Eight of the *H. pylori* RMSs are completely absent from both strain 26695 and strain J99. No RMSs are present in both of the strains as an intact DMT-RE pair. Of two RMSs, in both strains only the DMT is active. This leaves 14 RMS that differ between the two strains. Of these, three DMTs are present in both strains, but differ with respect to the presence of the RE: two are accompanied by their corresponding RE in strain 26695 and one in J99. The other 11 DMTs are present only in one of the two strains: seven DMTs are present only in J99, four of them with a functioning RE, and four DMTs are present only in 26695, two with an intact RE.

Definition of palindrome avoidance in H. pylori. Of all 256 possible tetrameric combinations of A, C, G and T, 253 have an observed/expected (obs/exp) ratio between 0.4 and 1.6 with a distribution around 1.0 in the genomes of both strains (not shown). This was considered the normal variation among tetramers. The remaining 3 tetramers, the palindromes GTAC, ACGT, and TCGA, have extreme obs/exp ratios below 0.2 in both strains, which is considered significant palindrome avoidance. GTAC has obs/exp ratios of 0.15 and 0.10 in strains J99 and 26695, respectively, but this sequence is, to our knowledge, not recognized by any RMS that has been described in *H. pylori* at present. ACGT is the recognition site of Hpy99XI. This RMS is absent from strain 26695, but in J99, an intact DMT with an inactive RE is present (Table 1). TCGA is recognized by Hpy V, which is absent from both sequenced *H. pylori* strains (Table 1).

Of the 1024 possible pentamers, only 4 (again all palindromes) have an obs/exp ratio below 0.2. Two of these, ACAGT and ACTGT, are recognized by HpyCH4 III (recognition site: ACNGT). This RMS is completely absent from both sequenced *H. pylori* strains. The

TABLE 1. The *H. pylori* Restriction modification systems. All *H. pylori* RMSs with their recognition sites, their presence and activity in the two sequenced *H. pylori* strains 26695 and J99, the obs/exp ratios for each recognition site and the ratio of the two obs/exp ratios. Obs/exp ratios with palindrome avoidance are shown in **bold**, as are the two deviant strain-ratios. The table is assembled with data from references (2005;Lin *et al.*, 2001;Lin *et al.*, 2004) and this study.

Name	Recognition site	26695			J99			26695 / J99 strain-ratio
		DMT	RE	obs/exp	DMT	RE	obs/exp	
Hpy99XI	ACGT	inact.	inact.	**0.09**	**yes**	inact.	**0.15**	**0.59**
HpyAI/iceA/Hpy99X	CATG	**yes**	inact.	1.25	**yes**	no	1.29	0.96
M.HpyAVIA/Hpy99V	CCTC	**yes**	inact.	0.68	**yes**	no	0.69	0.99
Hpy99VIII	CCGG	inact.	inact.	1.45	**yes**	inact.	1.51	0.96
HpyAIII/Hpy99VI	GATC	**yes**	**yes**	0.85	**yes**	inact.	0.87	0.97
M.HpyAVIII/Hpy99III	GCGC	**yes**	no	0.61	**yes**	**yes**	0.62	0.98
Hpy 178 VII	GGCC	no	no	0.42	no	no	0.41	1.04
Hpy V	TCGA	no	no	**0.12**	no	no	**0.13**	0.92
HpyCH4 V	TGCA	no	no	1.16	**yes**	no	1.12	1.03
HpyCH4 III	ACNGT	no	no	**0.27**	no	no	**0.28**	0.95
HpyCH4 II	CTNAG	no	no	0.60	no	no	0.60	1.00
HpyAIV/Hpy99IX	GANTC	**yes**	**yes**	0.55	**yes**	inact.	0.55	1.00
Hpy188 I	TCNGA	inact.	inact.	0.59	inact.	inact.	0.56	1.06
Hpy99IV	***CCNNGG***	no	no	0.67	**yes**	**yes**	0.65	1.03
HpyAIX	GTNNAC	**yes**	inact.	0.30	inact.	inact.	0.32	0.95
Hpy178 III	TCNNGA	inact.	inact.	0.60	**yes**	**yes**	0.57	1.05
HpyF10 VI	GCN(7)GC	no	no	n.d.	no	no	n.d.	n.d.
Hpy99I	CGWCG	no	no	0.56	**yes**	**yes**	0.55	1.01
Hpy99II	GTSAC	no	no	**0.10**	**yes**	**yes**	**0.13**	**0.78**
HpyAII	GAAGA	**yes**	**yes**	1.22	inact.	no	1.23	0.99
Hpy178 V	GGATG	no	no	0.56	no	no	0.55	1.02
M.HpyAVII	ATTAAT	**yes**	inact.	0.72	inact.	inact.	0.67	1.08
HpyAV/HgaI	?	**yes**	**yes**	n.d.	no	no	n.d.	n.d.
BcgI B/S/R-M	?	inact.	inact.	n.d.	inact.	inact.	n.d.	n.d.

other two pentamers, GTCAC and GTGAC, are recognized by Hpy99II RMS (recognition site: GTSAC). Hpy99II forms a complete intact RMS in strain J99 and is absent from strain 26695.

Comparison of palindrome avoidance between the strains. The difference in palindrome avoidance between the two sequenced genomes is expressed as the ratio of the obs/exp ratios of the two strains (strain-ratio). As expected, the recognition sites without palindrome avoidance all have a strain-ratio around 1.0 (0.95 – 1.08, table 1), indicating that there are no significant differences in the number of occurrence of these recognition sites between strains with and without the RMS. The recognition sites of two RMSs that are absent from both the genomes are also avoided and have ratios of 0.95 and 0.92, which is comparable to the sites without palindrome avoidance. But GTSAC, the recognition site of Hpy99II, which is absent from 26695 and intact in J99, has a strain-ratio of 0.78. ACGT, the recognition site of Hpy99XI, which is inactivated in 26695 and has an active DMT in J99, has

a strain-ratio of 0.59. For both recognition sites, the palindrome avoidance is stronger in the strain *not* possessing the corresponding DMT.

Discussion

The findings presented here contradict the theory of selective pressure of a genomic RE as the single cause of palindrome avoidance. This theory predicts that strains possessing a certain RMS have stronger palindrome avoidance than strains that do not. In the two *H. pylori* strains that we compared, some RMSs do not cause palindrome avoidance at all whereas two other palindromes are avoided, despite the absence of a known *H. pylori* RMS. More importantly however, the RMSs that are present in only one of the two strains do not cause stronger avoidance in that strain than in the other. Two mechanisms could potentially mask the effect of restriction-driven palindrome avoidance in our study. First, RMSs can be relatively unstable (Aras *et al.*, 2001;Gelfand & Koonin, 1997), as is confirmed by the large number of inactive or partially inactive RMSs in *H. pylori* (table 1). The activity of the *H. pylori* RMSs might change too fast to allow an individual strain to adapt to a specific "pattern" of RMSs, and thus palindromes are avoided irrespective of the presence of an RMS at a specific time point. Alternatively, it could be that the genomic fragments with avoided palindromes are exchanged with strains without the corresponding RMS. Indeed, *H. pylori* has a panmictic population structure with frequent horizontal gene transfer (Go *et al.*, 1996; Salaun *et al.*, 1998; Suerbaum *et al.*, 1998; Achtman *et al.*, 1999; Falush *et al.*, 2001). As long as there is no strong selective force in strains without a specific RMS against palindrome avoidance, it is possible that sequences with palindrome avoidance are stably embedded in all strains of the species, as long as a subset of strains contains the RMS. However, both theories still do not explain why there is a tendency to a *stronger* palindrome avoidance in strains without a DMT than in strains with a DMT. We considered the possibility that this is a statistical artifact. Because of the palindrome avoidance, the number of appearances of the oligonucleotide sequence is lower than that of non-avoided oligonucleotides. Because of this lower number, random differences between strains are more easily translated into a large strain-ratio for avoided oligonucleotides than for non-avoided oligonucleotides. However, the strain-ratio of 0.59 for ACGT is based upon a fairly large number of occurrences, 282 and 500 in strains 26695 and J99, respectively. For a comparison, we scored the strain ratios of all 4,096 possible hexameric oligonucleotides, most of which are present in lower numbers than ACGT in *H. pylori*. Strain-ratios of a similar magnitude occur only in hexameric sequences that are at least 10 times less abundant than ACGT (not shown), indicating that the difference

found for ACGT is unlikey to be a coincidence. The second possible explanation is that the DMT of HpyXI exerts a protective effect on the recognition site, which reduces the selective pressure for palindrome avoidance. This hypothesis also seems contradicted by the many inactive remnants of DMTs that are present in both *H. pylori* genomes, suggesting that DMTs are not very beneficial to their host after the loss of the corresponding RE. Both explanations are therefore not completely satisfactory.

Our results show that palindrome avoidance is not stronger in strains with a corresponding RMS than in other strains, and therefore do not support the hypothesis that RMSs increase palindrome avoidance in *H. pylori*. These results need to be confirmed by similar comparisons within other species.

HpyCH4 V. New England Biolabs inc. & Vanderbilt University patent 6133009 (2005)
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Chapter 7:

Summary & general conclusions

The aim of this thesis was to study natural transformation and its effect upon the population structure of *Helicobacter pylori*. As is explained in Chapter 1, the *H. pylori* population structure is almost panmyctic, which means that genetic markers recombine at a frequency that is high enough to eliminate the effect of clonal descent and to generate a linkage equilibrium between alleles at different loci [1, 3, 4, 9, 10]. The above is, however, merely inferred from phylogenetic data, and descriptions of actual recombination events are very scarce. In chapter two, we study the occurrence of recombination during co-habitation of two strains in one stomach. In one single DNA locus we were able to demonstrate that homologous recombination events had occurred in both of the strains. We show that *H. pylori* can exchange DNA fragments of at least 1.7 kb, long enough to contain one or more complete genes, and confirms the conclusions from phylogenetic studies. This emphasizes the importance of recombination in *H. pylori*.

Recombination between different bacteria is also called "horizontal gene transfer". The main mode of horizontal gene transfer used by *H. pylori* is natural transformation. Natural transformation is characterized by active uptake of extracellular DNA over the cell wall of the transforming bacterium. Subsequently, the imported DNA is integrated into the genome by homologous recombination or, if the DNA is a plasmid, by recircularization. The structural core of the *H. pylori* DNA translocation apparatus is related to the type IV secretion system [5, 6]. In Chapter 3, we describe a search in the *H. pylori* genomic sequence for genes involved in natural transformation. The search is based upon homologies with known transformation genes in other species. In these experiments we show that mutants in the *H. pylori dprA* gene are shown to be severely impaired in transformation with both chromosomal DNA and plasmids, similar to *recA* mutants. The latter is remarkable, because in *H. influenzae* plasmid transformation occurs independent from both *recA* [8] and *dprA* [7]. Apparently, *dprA* is necessary only during recombination-dependent processing of plasmids and not during recombination-independent processing. In Chapter 4, the function of DprA is studied in more detail. The DprA protein has been suggested to be involved in the protection of incoming DNA during natural transformation [2]. However, members of the *dprA/smf* gene family can be detected in virtually all bacterial species, except for some obligate intracellular pathogens and symbionts (this thesis). This means that DprA must have a broader function in DNA repair or recombination, and/or that DprA of natural competent species have a different function. In chapter four we show that an *E. coli dprA/smf* homolog is in fact able to partially restore competence in a *H. influenzae dprA* mutant, which shows that *dprA/smf* genes from competent and non-competent species are interchangeable. Subsequently, experiments were

performed to try to unravel the "normal" function of DprA in *E. coli*. However, no phenotype of such mutants regarding transformation, Hfr-conjugation, recombination and DNA repair were observed. Therefore, the basic function of *dprA/smf* remains unclear.

In Chapter 5, a new search for genes necessary for natural transformation in *H. pylori* is described. In contrast to chapter three, homology with genes from other competent species is not used as a search tool. Instead, a bank of random mutants is screened for mutants that are not able to transform. This results in the identification of a novel, *Helicobacter*-specific competence gene (*comH*) whose function is essential for transformation of *H. pylori* with chromosomal DNA fragments as well as with plasmids. Unlike other transformation genes of *H. pylori*, *comH* does not belong to a known family of orthologous genes. The *comH* gene codes for a protein with an N-terminal leader sequence. Because the predicted mature ComH protein is highly charged, the ultimate location of ComH is the periplasm or the extracellular medium. Antibodies against ComH were generated, but unfortunately no expression of ComH could be detected (Smeets *et al*., unpublished results). The *comH* gene is also a rather unique gene, it is present in both highly competent and less efficiently transforming *H. pylori* strains, but apart from *H. pylori* it is only found in *Helicobacter acinonychis*, a species that is highly related to *H. pylori* and can also be naturally transformed, but not in other bacteria such as *H. felis* and *H. mustelae*. Moreover, no significant homologs of *comH* are identified in currently available databases of bacterial genome sequences. It is not yet clear what the function of ComH in the transformation process is. The putative periplasmic location of the protein suggests a role in the uptake of DNA. In accordance with this, *comH* mutants can be successfully electrotransformed.

In Chapter 6 we study the phylogenetic phenomenon called "palindrome avoidance". Palindrome avoidance is the name for the observation that palindromic oligonucleotide sequences are sometimes much less abundantly present than can be explained by coincidence. According to the *restriction hypothesis*, this is caused by restriction-modification systems (RMSs). RMSs often use short palindromes as their recognition sequence; therefore palindromes are at a higher risk of being cut by such enzymes than other oligonucleotides. A reduced presence of palindromes could reduce the danger of potentially lethal restriction for the bacterium. To test the restriction hypothesis for palindrome avoidance, we compared the frequency of the presence of RMSs to the avoidance of their recognition sequences in the two published *H. pylori* genomic sequences. In contrast to the hypothesis, palindrome avoidance in *H. pylori* is not stronger in *H. pylori* strains with the corresponding RMS than in strains without. This can be explained by the frequent horizontal gene transfer in *H. pylori*.

In this thesis the importance of natural transformation for *H. pylori* is underscored, and a contribution to the understanding of this fascinating process is made.

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Nederlandstalige samenvatting

Bacteriën kennen geen geslachtelijke voortplanting, ze hebben altijd één "ouder" in plaats van twee. Ze kunnen dus tijdens de voortplanting niet kruisen. Om toch erfelijke eigenschappen te kunnen uitwisselen hebben ze andere methoden. De maagbacterie *Helicobacter pylori* kan dit bijvoorbeeld doen door DNA dat in de omgeving aanwezig is ("vrij DNA") op te nemen, een proces dat transformatie heet. Dit vrije DNA kan afkomstig zijn van een soortgenoot met net iets andere eigenschappen, en deze eigenschappen gaan dan over op de transformerende bacterie. Het doel van dit promotie onderzoek was ten eerste het in kaart brengen van het systeem dat het DNA over de celwand van *H. pylori* heen kan transporteren, en ten tweede het effect van transformatie op de soort *H. pylori* te onderzoeken. In hoofdstuk twee worden daarom twee *H. pylori*-stammen beschreven die langere tijd samen in één maag hebben geleefd. Onderzoek aan deze stammen maakte duidelijk dat er verschillende stukken DNA van de ene op de andere stam waren overgegaan. Het grootste stuk DNA dat in één keer van de ene naar de andere stam is gegaan moet tenminste 1.700 baseparen lang geweest zijn, voldoende om minimaal één intact gen over te dragen.

In hoofdstuk drie wordt in het genoom van *H. pylori* gezocht naar genen die homologie vertonen met (=lijken op) genen die in andere bacteriesoorten een rol spelen bij transformatie. Dit levert een aantal genen op. Proeven met *H. pylori* stammen ("mutanten") waarin deze genen kapot gemaakt zijn, levert één mutant op die niet meer kan transformeren. Hiermee is aangetoond dat het betreffende gen, *dprA* genaamd, noodzakelijk is voor *H. pylori* om te transformeren. Onderzoek aan genomen van andere bacteriën maakt duidelijk dat vrijwel iedere bacteriesoort een soortgelijk gen heeft, maar wat het gen precies doet is onbekend.

In hoofdstuk 4 wordt de rol van de *dprA* genfamilie in meer detail onderzocht. Hierbij wordt ten eerste bewezen dat een *dprA* van de bacteriesoort *Escherichia coli* de rol van *dprA* in de soort *Haemophilus influenzae* kan overnemen, en dat *dprA* genen in verschillende soorten dus eenzelfde functie hebben. Ondanks verdere proeven aan *dprA* blijft de exacte functie van *dprA* onbekend.

In hoofdstuk 5 wordt opnieuw gezocht naar genen die nodig zijn voor transformatie. In tegenstelling tot hoofdstuk 3 niet door te kijken naar genen die lijken op bekende 'transformatiegenen' uit andere bacteriesoorten, maar door willekeurig genen in *H. pylori* bacteriën uit te schakelen en te kijken welke uitschakeling leidt tot een mutant die niet meer in staat is tot transformatie. Dit levert opnieuw een transformatiegen op: *comH*. In tegenstelling

tot *dprA* hebben andere bacteriesoorten geen op *comH* gelijkende genen: *comH* is uniek voor *H. pylori*. Het gen codeert waarschijnlijk voor een eiwit dat aan de buitenzijde of in de celwand van de bacterie is gelegen. Dit suggereert dat het betrokken is bij het proces van DNA transport door de celwand.

In hoofdstuk 6 wordt het fenomeen 'palindrome avoidance' onderzocht. Palindrome avoidance betekent dat palindromische DNA-sequenties in bacteriën minder vaak voorkomen dan je zou verwachten op grond van kansberekening. De reden hiervoor is onbekend, maar één van de mogelijke verklaringen is gelegen in enzymen die DNA kapotknippen: deze knippen meestal in één specifieke sequentie en dat is vrijwel altijd een palindromische sequentie. Omdat het knippen van DNA potentiëel dodelijk is voor de cel, zou dat een reden kunnen zijn waarom palindromen erg weinig voorkomen. Om deze hypothese te testen zijn twee *H. pylori* stammen, die verschillende DNA-knippende eiwitten bevatten, onderzocht op palindrome avoidance. Er was echter geen correlatie tussen de aanwezigheid van enerzijds een DNA-knippend enzym en anderzijds de palindrome avoidance van het palindroom dat hierdoor geknipt wordt. De hypothese is dus waarschijnlijk onjuist in *H. pylori*.

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